

Title	Macrophage polarisation: the impact of M1 versus M2 polarisation on host innate immune responses to bacterial infection
Authors	Foley, Niamh M.
Publication date	2015-12
Original Citation	Foley, N. M. 2015. Macrophage polarisation: the impact of M1 versus M2 polarisation on host innate immune responses to bacterial infection. MD Thesis, University College Cork.
Type of publication	Doctoral thesis
Rights	© 2015, Niamh M. Foley. - https://creativecommons.org/licenses/by-nc-nd/4.0/
Download date	2023-05-05 10:54:08
Item downloaded from	http://hdl.handle.net/10468/11410

Macrophage Polarisation: the impact of M1 versus M2 polarisation on host innate immune responses to bacterial infection

A thesis submitted to the National University of Ireland for the MD degree examination of:

Niamh Foley MB BCh BAO BA MRCSI



SUPERVISORS:

Prof HP Redmond

Dr JH Wang

AFFLIATION:

Department of Academic Surgery, National University of Ireland, Cork, Ireland.

SUBMITTED:

December 2015

Acknowledgements

I would like to take this opportunity to thank everyone who helped and supported me in writing this research MD. I would like to express my extreme gratitude to Professor Redmond, who guided me through this. His mentorship, patience and encouragement were invaluable, not only during my time in research but also in my fledgling surgical career. I am particularly grateful to Dr Wang for his valuable and constructive suggestions during the planning and execution of this research and especially for always being on hand to answer my innumerable questions. My special thanks are extended to Qiondi Wu and Siobhan Blankson for their laboratory assistance.

I would like to thank my colleagues in the research laboratory who, over time became friends, especially Gaitri Sadacharam who guided me through my first few months and Caitriona Lyons, who was always available to answer any number of enquiries. A special acknowledgement and thanks is reserved for Grace O'Callaghan, without whose encouragement and support, I would not have completed this thesis. Ultimately, I would like to acknowledge the support provided by my family, who have, steadfastly, been by my side.

Table of Contents

Declaration	i
Acknowledgements	ii
Abstract	vii
Abbreviations	ix
List of Publications	xiv

Chapter 1: Introduction

1.1	Sepsis	1
1.1.1	Definition	1
1.1.2	Incidence and mortality of sepsis	4
1.1.3	Risk factors in sepsis	5
1.1.4	Aetiology of sepsis	6
1.1.5	Bacterial sepsis	6
1.1.6	Classification of bacteria	6
1.1.7	Aetiology of bacterial sepsis	7
1.1.8	Pathophysiology of sepsis	8
1.1.9	Treatment of sepsis	8
1.1.10	Antibiotic therapy	9
1.1.11	Early goal directed therapy	9
1.1.12	Immune modulation	9

1.2	Immune response to sepsis	10
1.2.1	Adaptive immune response	11
1.2.2	Innate immune response to sepsis	11
1.3	Macrophages	13
1.3.1	M1 macrophages (classically activated)	15
1.3.2	Signaling in M1 macrophages	15
1.3.3	M2 macrophages	19
1.3.4	Role of macrophages in the immune system	21
1.3.5	Macrophage polarisation and its role in sepsis	22
1.4	Signaling pathways involved in sepsis	24
1.4.1	Toll-like receptors	24
1.4.2	TLR4	24
1.4.3	TLR2	25
1.4.4	TLR signalling in sepsis	25
1.4.5	MyD88 dependent pathway	26
1.4.6	P38 MAPK signalling in sepsis	27
1.5	Endotoxin Tolerance	30
1.5.1	The role of endotoxin tolerance in sepsis	30
1.5.2	Similarities between endotoxin tolerance and the M2 macrophage polarisation pathway	31
1.6	Summary	33

Chapter 2: Materials and Methods	46
2.1 Reagents	46
2.2 Bacteria	47
2.3 Isolation of murine bone marrow-derived macrophages	47
2.4 Polarisation of M1 and M2 phenotypes	48
2.5 Stimulation of polarised M1 and M2 macrophages	48
2.6 Enzyme-linked immunosorbant assay	48
2.7 Western blot analysis	49
2.7.1 Sample preparation	49
2.7.2 Bicinchoninic acid (BCA) assay	49
2.7.3 Sodium Dodecyl Sulfate Polyacrylamide Gel	
Electrophoresis (SDS-PAGE) and Western blotting	50
2.8 Statistical analysis	51
 Chapter 3: Results	 52
3.1 Cytokine Results	52
3.1.1 IL-6 secretion in naïve and polarised macrophages	53
3.1.2 IL-10 secretion in naïve and polarised macrophages	56
3.1.3 IL-12 secretion in naïve and polarised macrophages	59
3.1.4 TGF- β secretion in naïve and polarised macrophages	62
3.1.5 TNF- α secretion in naïve and polarised macrophages	67

3.2 Western Blot Results	71
3.2.1 MAPK signalling in naïve and polarised macrophages	71
3.2.2 NF- κ B signaling in naïve and polarised macrophages	77
 Chapter 4: Discussion	 91
Chapter 5: Conclusion and Future Directions	99

ABSTRACT

Background and Aim

Infection is a global burden causing millions of deaths per annum worldwide. In the US sepsis is the tenth leading cause of death with the mortality associated with severe sepsis estimated at 30-50%. Innate immunity is a generic response mediated by the host to protect it from bacterial infection. The recognition of foreign microbes leads to activation of pattern recognition receptors and recruitment of macrophages. In acute bacterial infections, activated macrophages polarised to M1 or M2 states play a major role in the host cytokine response which drives the immune response until the host has overcome the invading microbial pathogen. The aim of this study was to 1) to characterise the cytokine profile of M1 and M2 polarised macrophages 2) to investigate the changes in the cytokine profile of polarised macrophages in response to bacterial stimulation 3) to examine the role of the MAPK and NFκB signalling pathways in the response of naïve and polarised macrophages to bacterial infection.

Results: (i) polarisation of macrophages to an M1 state resulted in a higher secretion of pro-inflammatory cytokines (IL-6, IL12p70 and TNF-α.). (ii) following bacterial stimulation M1 polarised macrophages had reduced pro-inflammatory cytokine release. (iii) M1 polarised macrophages have reduced MAPK and NFκB signalling as detected by western blot analysis.

Conclusion

Following bacterial stimulation M1 polarised macrophages had reduced pro-inflammatory cytokine release which may in part be due to reduced MAPK and NFκB signalling. This data suggests that M1 polarisation states may play important roles in an endotoxin tolerant phenomenon in acute bacterial sepsis.

Abbreviations

PAMPs	Pathogen Associated Molecular Patterns
PRRs	Pathogen Recognition Receptors
TLRs	Toll like Receptors
NOD	Nuclear Oligomerization Domain
NLRs	Nod like Receptors
CLRs	C-type Lectin Receptors
RIG-I	Retinoic acid-inducible gene-I
RLRs	RIG-I like Receptors
LPS	Lipopolysaccharide
IL-1R	Interleukin-1 Receptor
LRRs	Leucine Rich Repeats
RNA	Ribonucleic acid
dsRNA	double stranded RNA
ssRNA	single stranded RNA
TIR	Toll/IL-1R domain
TIRAP	TIR domain containing adapter protein

TICAM	TIR-containing adaptor molecule-1
MyD88	Myeloid differentiation primary response 88
IRAK	IL-1 receptor kinase
TRAM	TRIF related adapter molecule
TRAF	Tumour Necrosis Factor Receptor Associated Factor 3
TAK-1	TGF- β -activated kinase
IKK	Inhibitor of kappa B kinase- β
I κ B α	Inhibitory kappa B alpha
NF κ B	Nuclear factor kappa B
JNK	c-Jun N-terminal Kinase
ERK	Extracellular signal related kinase
MAPK	Mitogen-activated protein kinases
RIP-1	Receptor interacting protein 1
TRADD	Tumour necrosis factor receptor type1-associated Death domain protein
IRF3	Interferon regulatory factor 3
BMDM	Bone Marrow-Derived Macrophage
CARD	Caspase activation and recruitment domain

TNF	Tumour Necrosis Factor
IFN	Interferon
CARDIF	CARD adapter inducing IFN β
DC	Dendritic cell
MMP	Metalloproteinases
FCS	Foetal calf serum
PMA	Phorbol 12-myristate 13-acetate
Sp	species
APC	activated protein C
TAK-242	small molecule specific inhibitor of TLR4, Resatorvid
PGN	peptidoglycan
LTA	lipoteichoic acid
DAMPs	Danger associated molecular patterns
iNOS	inducible nitric oxide synthase
NO	nitric oxide
IRF	interferon regulatory factor
PAF	platelet aggregating factor

PGE2	prostaglandin
MCP-1	monocyte chemoattractant protein-1
COX-2	cyclo-oxygenase 2
MyD88	myeloid differentiation factor 88
MAL	MyD88 adaptor like protein
TRIF	TIR domain containing adaptor protein inducing IFN- β
STAT	signal transducers and activators of transcription
SOCS	suppressors of cytokine signaling
IFN	interferon
RIP	receptor-interacting protein
TNF	tumour necrosis factor
KLF4	Kruppel like factor 4
M-CSF	macrophage colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
Relm- α	resting like molecule α (also known as Fizz-1)
Ym-1	chitinase 3-like 3
TGM2	transglutaminase 2

Arg-1

arginase 1

Publications/Presentations/Abstracts

**Association of Surgeons of Great Britain and Ireland International Surgical Congress,
Harrogate, United Kingdom. April 22nd – 24th 2015, Manchester**

Diminished MAPK signaling in M1 macrophages exposed to bacterial stimulation

27th European Congress of Surgical Infections, Vienna, 5th – 7th June 2014

M1 polarised macrophages develop an endotoxin tolerant like phenomenon in response to
bacterial stimulation

**49th Congress of the European Society for Surgical Research, Budapest, Hungary. 21st –
24th May 2014**

M1 polarised macrophages develop an endotoxin tolerant like phenomenon in response to
bacterial stimulation

**Association of Surgeons of Great Britain and Ireland International Surgical Congress,
Harrogate, United Kingdom. April 30th – 2nd May 2014**

M1 polarised macrophages develop an endotoxin tolerant like phenomenon in response to
bacterial stimulation

Short Papers of Distinction Session

Sir Peter Freyer Annual Meeting, Galway, September 5th and 6th 2014

Diminished MAPK signaling in M1 polarised macrophages exposed to bacterial stimulation

Plenary session

Sylvester O'Halloran Meeting, Limerick, Feb 28th and March 1st 2014

M1 polarised macrophages develop an endotoxin like phenomenon in response to bacterial stimulation

Plenary Session

Sir Peter Freyer Meeting, Galway, 6th and 7th September 2013

Macrophage Polarisation

Foley, N. M., J. H. Wang, and H. P. Redmond. "M1 Polarised Macrophages Develop an Endotoxin Tolerance-Like Phenomenon in Response to Bacterial Stimulation." *British Journal Of Surgery*. Vol. 102. 111 River St, Hoboken 07030-5774, NJ USA: Wiley-Blackwell, 2015.

Foley, N. M., J. H. Wang, and H. P. Redmond. "M1 polarised macrophages develop an endotoxin tolerance-like phenomenon in response to bacterial stimulation." *International Journal of Surgery* 12 (2014): S15.

Current knowledge and future directions of TLR and NOD signaling in sepsis

NM Foley, J Wang, HP Redmond, JH Wang

Military Medical Research 2015 Jan 7;2:1 DOI 10.1186/s40779-014-0029-7

Chapter 1

Introduction

Sepsis

“The presence in tissues of harmful bacteria and their toxins typically through infection of a wound”

-Oxford English Dictionary

1.1 Sepsis

1.1.1 Definition

Sepsis, an age old enigma, was first recounted by Homer in the Iliad.¹ The word is of Greek origin, literally meaning “I rot”. Sepsis was described by Hippocrates as the process by which “flesh rots, swamps generate foul airs and wounds fester”.² Interestingly, Galen was one of the first to consider sepsis a necessary event in order to allow wound healing,³ but it was not until the research of Louis Pasteur that the definitive link between ‘germs’ and infection was demonstrated.⁴ Sepsis is defined as the probable or documented presence of microbial infection together with systemic manifestations of infection.⁵ It is a potentially life-threatening condition, ranking in the top 10 causes of death.⁶ Sepsis occurs following a breach of integrity of any one of the host barriers, e.g. physical, immunological or direct penetration of the pathogen into the bloodstream.⁷ Physical barriers include the gastrointestinal, respiratory, genitourinary and integumentary systems.

Several different terms have been used to describe the overwhelming inflammatory response associated with acute infections including sepsis, septicaemia and septic shock. This can lead to confusion in national and international reporting of sepsis. As a direct result of these confusing terms, a consensus meeting was convened in 1992. At this conference, the American College of Chest Physicians/Society for Critical Care Medicine (ACCP/SCCM)⁸ decided, following collaboration and consensus, upon a definition of sepsis as the presence of at least two out of four criteria listed in the table below as well as probable or documented infection.

Definition of Sepsis	The presence of two out of four of these criteria
Temperature	> 38° or < 36° Celsius
Heart rate	> 90 beats per minute
Hyperventilation	> 20 respirations per minute or PaCO ₂ < 32 mmHg
White cell count	> 12,000 cells/μL or < 4,000 cells/μL

Table 1. Definition of systemic inflammatory response. Must incorporate at least two of the four criteria.

These criteria were subsequently updated in the Society of Critical Care Medicine/European Society of Intensive Care Medicine/American College of Chest Physicians/American Thoracic Society/Surgical Infection Society (SCCM/ESICM/ACCP/ATS/SIS) International Sepsis Definitions Conference in 2001.⁹ (Table 2) The range of criteria included highlights the distinct difficulty that has existed for years with the definition of sepsis.

Infection ¹ : Documented or suspected and some of the following ²	
General Parameters	Fever (core temperature >38.3°C) Hypothermia (core temperature <36°C) Heart rate >90 beats per minute or >2SD above the normal value for age Tachypnoea >30 breaths per minute Altered mental state Significant oedema or positive fluid balance (>20ml/kg over 24 hours) Hyperglycaemia (plasma glucose >110 mg/dL or 7.7 mmol/L in the absence of diabetes)
Inflammatory Parameters	Leukocytosis (white blood cell count >12,000/μL) Leukopaenia (white blood cell count <4,000/μL) Normal white blood cell count with >10% immature forms Plasma C reactive protein >2SD above the normal value Plasma procalcitonin >2SD above the normal value
Haemodynamic Parameters	Arterial hypotension ² (systolic blood pressure <90 mmHg, mean arterial pressure <70 or a systolic blood pressure decrease >40mmHg in adults or <2SD below normal for age) Mixed venous oxygen saturation >70% Cardiac index >3.5min ⁻¹ m ⁻² (3,4)
Organ Dysfunction Parameters	Arterial hypoxaemia ² (PaO ₂ /FiO ₂ <300) Acute oliguria (urine output <0.5ml/kg/hr or 45mM/L for at least 2 hours) Creatinine increase ≥0.5mg/dL Coagulation abnormalities (international normalised ratio >1.5 or activated partial thromboplastin time >60 seconds) Ileus (absent bowel sounds) Thrombocytopenia (platelet count <100,000/μL) Hyperbilirubinaemia (plasma total bilirubin >4mg/dL or 70mmol/L)
Tissue Perfusion Parameters	Hyperlactatemia (>3mmol/L) Decreased capillary refill or mottling

Table 2. Adapted from the Society of Critical Care Medicine/European Society of Intensive Care Medicine/American College of Chest Physicians/American Thoracic Society/Surgical Infection Society (SCCM/ESICM/ACCP/ATS/SIS) International Sepsis Definitions Conference in 2001.

¹. Defined as a pathological process induced by microorganisms.

². Values above 70% are normal in children and should therefore not be used as a sign of sepsis in newborns.

³. Values of 3.5-5.5 are normal in children and should therefore not be used as a sign of sepsis in newborns or children.

⁴. Diagnostic criteria for sepsis in the paediatric population include signs and symptoms of inflammation plus infection with hyper or hypothermia, rectal temperature >38.5°C or >35°C, tachycardia (may be absent in hypothermic patients) and at least one of the following indications of altered organ function: altered mental status, hypoxaemia, elevated serum lactate and bounding pulses

1.1.2 Incidence and mortality of sepsis

Sepsis is a systemic, deleterious host response to infection leading to severe sepsis and septic shock. It is an important, but perhaps overlooked public health problem. Studies suggest that acute infections can often exacerbate pre-existing chronic conditions or result in new chronic diseases. In the US severe sepsis is the tenth leading cause of death, similar in number to those dying from acute myocardial infarction. The risk of dying from sepsis is rising year on year,^{10,11} and mortality associated with severe sepsis is estimated at 30-50%.^{12,13}

1.1.3 Risk factors in sepsis

Risk factors for sepsis include those outlined in table 3.

Table 3: Risk factors associated with sepsis

Risk Factors for Sepsis	Risk Factors associated with mortality in cases of severe sepsis
Extremes of age	Acute renal failure
Chronic disease	Shock
Severe injury	Smoking
Pre-existing infection	Use of mechanical ventilation
Organ dysfunction	Dementia
Extended hospitalisation	Advanced age
Immune compromise	Chronic liver disease
	Cardiac failure

Treatment in the initial hours after the onset of sepsis significantly influences outcome.

Variability exists in reported severe sepsis mortality, with a rate of 8.6% (range 0.9 – 18.2%) across 188 Hospitals in the US recently described,¹⁴ however a further study reported higher in-hospital mortality rates ranging from 14.7% to 29.9%.¹⁵

Sepsis is also a global financial burden.¹⁶ The costs associated with sepsis care are mainly related to the price of targeted new therapies such as activated protein C, which costs \$27,936 per life year gained¹⁷, technologies and also the increasing charges for fixed costs. Angus and co-workers estimated the cost of sepsis treatment in the United States in 2001 at \$16.7 billion annually.¹⁸ This figure had risen to \$24.3 billion by 2007,¹⁹ highlighting the significant financial burden accompanying sepsis and the need for new effective low cost therapies.

1.1.4 Aetiology of Sepsis

Over 90% of cases of sepsis are caused by bacteria. Much less commonly, in approximately 6% of cases, fungal causes are implicated; viral and parasitic causes are rare.⁶ The aetiology remains elusive in approximately 30% of cases of sepsis, due to the inability to isolate the offending pathogen.

1.1.5 Bacterial sepsis

Bacterial sepsis is a symptomatic bacteraemia with or without end organ dysfunction.

Confirmation of microbial presence relies on culturing the pathogen from tissue samples e.g. pus or blood, in a dedicated microbiology laboratory. This process takes a minimum of 24 hours, in order to allow growth of the organism, but is only positive in approximately 50% of cases⁶ making targeted narrow spectrum antibiotic therapy challenging.

1.1.6 Classification of bacteria

Hans Christian Joachim Gram, a Danish bacteriologist working in Berlin, devised a method in 1884, for dividing bacteria into two main groups: bacteria that have the ability to retain an initial crystal violet stain are termed gram-positive whereas those that are decolourised and stain red with carbol fuchsin are termed gram-negative.²⁰ He did this by examining lungs from patients who had died from pneumonia. The two groups behave differently; 90-95% of Gram negative organisms are pathogenic, whereas many gram positive organisms are not pathogenic. However, it remains a fact, that the vast majority of severe sepsis cases are as a result of infection with gram-positive bacteria.

Table 4. Common pathogenic gram positive and gram negative bacteria

Gram positive bacteria	Gram negative bacteria
<i>Staphylococcus sp</i>	<i>Salmonella typhi</i>
<i>Enterococcus sp</i>	<i>Haemophilus influenza</i>
<i>Streptococcus sp</i>	<i>Neisseria meningitidis</i>
<i>Bacillus sp</i>	<i>Escherichia coli</i>
<i>Clostridium sp</i>	<i>Klebsiella sp</i>
<i>Actinomyces</i>	<i>Enterobacter sp</i>
<i>Mycobacterium</i>	<i>Pseudomonas sp</i>
<i>Mycoplasma</i>	<i>Proteus sp</i>
<i>Streptomyces</i>	<i>Acinetobacter sp.</i>

1.1.7 Aetiology of bacterial sepsis

The incidence of gram-positive bacterial sepsis has increased over time and is now almost as common as gram-negative sepsis.⁶ In a 2006 European multicentre study of septic patients admitted to intensive care units, a respiratory source (68%) of sepsis was most common in 68% of cases, followed by an intra-abdominal source in 22% of cases.²¹ The same study revealed the most common isolate as *Staphylococcus aureus*, followed by *Pseudomonas species* and *Escherichia coli*. The Extended Prevalence of Infection in Intensive Care study (EPIC II), an international study of the prevalence and outcomes of infection in intensive care units also found a predominant respiratory source of sepsis (64%), with 62% of isolated bacteria identified as gram-negative microorganisms.²² The most commonly isolated gram-negative bacteria in that study were *Pseudomonas sp.*, *Escherichia coli* and *Klebsiella sp.*. *Staphylococcus aureus* was one of the most commonly isolated gram-positive bacteria

followed by *Staphylococcus epidermidis* and *Streptococcus pneumonia*. The same study, using multivariate analysis, found that gram-negative bacteria, namely *Pseudomonas*, *Enterococcus* and *Acinetobacter sp.*, were associated with a greater risk of in-hospital mortality. The increasing incidence of gram-positive infection is attributed to the increasing numbers of invasive procedures and the increasing risk of developing hospital acquired infections.²³

1.1.8 Pathophysiology of sepsis

The precise pathophysiology involved in sepsis will be dealt with further in the chapter, but in brief, death from sepsis results from an overwhelming inflammatory cascade causing end-organ damage and multi-organ failure. When acting appropriately, the inflammatory response deals effectively with the invading organism without causing tissue or end organ damage. It is a complex interplay between anti-and pro-inflammatory signals and in most individuals the body is able to balance these competing tasks.

1.1.9 Treatment of Sepsis

The mainstay of sepsis care has been early broad-spectrum antibiotics and early goal-directed therapy. The concept of immune modulation in the treatment of sepsis is one of significant interest and ongoing research. Unfortunately, thus far, no immune modulator has proven effective in clinical trials.

1.1.10 Antibiotic therapy

A delay in antibiotic treatment for bacteraemia has been shown to increase mortality.²⁴

Empiric antibiotic therapy should be commenced where a diagnosis of sepsis is suspected and after cultures have been taken. Selection of appropriate antibiotics depends on a number of different factors including: local antimicrobial guidelines, the suspected source of sepsis, whether the infection is likely to be community- or hospital-acquired, the presence of foreign bodies and the immune competency state of the patient among others. Once a pathogen is identified, often at least 24 hours after presentation, antibiotic therapy can be rationalised.

1.1.11 Early Goal Directed Therapy

This has been used for the treatment of severe sepsis and septic shock. Circulatory collapse in severe sepsis leads to an imbalance between tissue oxygenation and tissue oxygen requirements, resulting in shock. Global tissue hypoxia is a precursor for the development of organ damage, multi-organ failure and death.²⁵ The ‘golden hour’ is a window of opportunity that exists when aggressive management of physiological parameters can provide an outcome benefit.²⁶ Optimised cardiac preload, afterload and contractility improves survival in septic patients.²⁷ Aggressive fluid replacement therapy is the first line of treatment in maintaining cardiac output, however if blood pressure remains low despite fluid challenge then vasopressors are recommended.

1.1.12 Immune Modulation

Treatments directly targeting the immune response to sepsis have so far proven disappointingly expensive and ineffective, despite promising animal and preclinical results.

Recombinant human activated protein C (APC) is one such example of an immune modulator targeting severe inflammation in the treatment of adult and paediatric sepsis. APC has both anticoagulant and cytoprotective effects. The recombinant human activated protein C worldwide evaluation in severe sepsis (PROWESS) study group sought to prove a benefit in mortality rates in patients with severe sepsis who were treated with APC, however the outcome was that treatment with APC provided no mortality benefit at 28 or 90 days. A separate trial, the PROWESS-SHOCK trial sought to prove a benefit in mortality for patients with septic shock who were treated with APC, however, again there were no significant differences in mortality rates at 28 and 90 days.²⁸

Other studies have addressed TLR signaling in sepsis, including a TLR4 agonist TAK-242 which showed great promise in pre-clinical trials but did not show any efficacy in phase 3 clinical trials.^{29,30,31}

Recent studies have indicated a role for the high mobility group box 1 (HMGB1) as a late mediator in experimental sepsis. The potential role of HMGB1 inhibitors such as TSN-SS in the clinical management of human sepsis have produced promising results with TSN-SS attenuating late inflammatory response and improving cardiovascular function in Chinese cardiovascular patients (Wang et al., 2014). However, robust safety studies along with extensive preclinical toxicology studies are required before therapeutic intervention in human sepsis³².

1.2 Immune response to sepsis

Sir William Osler noted that death from sepsis resulted from the response of the body to systemic infection as opposed to the infection itself. This view was expanded on in the

1970's and is now a widely accepted concept.³³ Death in the first few days from sepsis is generally understood to be a result of hyper-inflammation driven by inflammatory cytokines, leading to multi-organ failure. People at the extremes of life are at increased vulnerability to infection. In countries with good healthcare infrastructure, 75% of deaths from sepsis occur in those aged 65 and older.³⁴ The immune system affords the host an opportunity to respond to pathogenic organisms and incorporates innate and adaptive immunity.

1.2.1 Adaptive Immune Response

Adaptive or acquired immunity is a separate, more sophisticated line of defence against pathogens as compared to the innate immune response. It is a learned and specific response to invading micro-organisms, not the generic response that is characteristic of innate immunity. Adaptive immunity is dependent on the “rearrangement of genes, antigen specific and requiring time for induction during primary challenges”.³⁵ It is based in the recognition of antigens. Adaptive immunity takes longer to respond and relies heavily on antigen presenting cells and the ability to recognise the offending antigen. B and T cells form the backbone of the adaptive immune response through the generation of immunoglobulins and the reaction of activated T cells directly against an antigen. The ability to differentiate what is self from what is foreign is fundamental to adaptive immunity. Allergic responses like hayfever and asthma are examples of the adaptive immune system attacking its own cells.³⁶

1.2.2 Innate immune response to sepsis

A person's survival is dependent on an innate immune system that can quickly recognise and respond to foreign pathogens such as bacterial and viral products. Innate immunity is a

generic response and is the first line of defence in protecting the host from pathogens³⁷. It is defined as being “dependent on germline genes, present at all times and functional during early primary infections but not increasing with repeated exposure”.³⁸ Animals detect invading microorganisms through a family of receptors called pathogen-associated molecular patterns (PAMPs)³⁹. PAMPs are detected by cells of the innate immune system, through pattern recognition receptors (PRRs), composed of four main families: toll-like receptors (TLRs), C-type lectin receptors, retinoic gene 1-like receptors and nucleotide binding oligomerisation domain-like receptors (NLRs).⁴⁰ This generic immune response allows for the detection of a finite number of molecules that are common and conserved in different microbes, e.g. lipopolysaccharide (LPS) or lipid A, which is common to all gram-negative bacteria, or lipoteichoic acid (LTA) common to gram-positive bacteria.

Macrophages, neutrophils and dendritic cells are all salient elements of the host innate immune response. These cells can directly or indirectly target pathogenic microorganisms through phagocytosis or by releasing substances such as cytokines, chemokines and other mediators. Phagocytosis by macrophages initiates the innate immune response. This inflammatory response to invading pathogens is characterised by the release of a variety of different signalling molecules including inflammatory cytokines. This vital step in the elimination of pathogens from the host can result in an overwhelming inflammatory response. Overproduction of pro-inflammatory mediators such as cytokines can lead to an amplified secondary response. This hyper-inflammatory state, with loss of normal immune homeostasis, can lead to organ damage and death.

During an infectious or inflammatory state, circulating peripheral monocytes are recruited into tissues where they differentiate into macrophages. Following this and dependant on the microenvironment present, macrophages can further differentiate into two main functional phenotypes or polarisation states; M1 or classically activated macrophages and M2 or alternatively activated macrophages.

1.3 Macrophages

Macrophages are important, essential, key components of the host innate and adaptive immune system and serve the purpose of initiating, maintaining and resolving the immune response to infection. They are members of the mononuclear phagocyte system and derive from the myeloid lineage. The spleen serves as a reservoir for immature monocytes⁴¹, and once mature, monocytes circulate haematogenously as peripheral blood mononuclear cells from between one to three days.

Historically, it has been accepted that monocytes are macrophage precursors, serving to replenish macrophages and DCs both in ordinary circumstances and in response to inflammation or infection.⁴² However more recent data has suggested that adult tissue macrophages are actually derived from embryonic progenitor cells that seed developing tissues in utero.⁴³ Notwithstanding this recent development, monocytes are still regarded as key players in replenishing tissue macrophages in the setting of inflammation, infection and tissue remodelling.

Macrophages were first described by Elie Metchnikof in the late 1800's,⁴⁴ have a widespread tissue distribution, e.g. liver, gut, lung, brain, etc. and display remarkable phenotypic heterogeneity. Tissue macrophages have a repertoire of receptors with the purpose of identifying invading organisms. They do this through pattern recognition receptors (PRRs) which identify molecular patterns such as pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs). The response of the macrophage varies depending on the nature⁴⁵ and magnitude⁴⁶ of the insult. Macrophages, following activation by a stimulus, can be split into two main polarisation profiles or phenotypes. M1 or classically activated macrophages and M2 or alternatively activated macrophages.

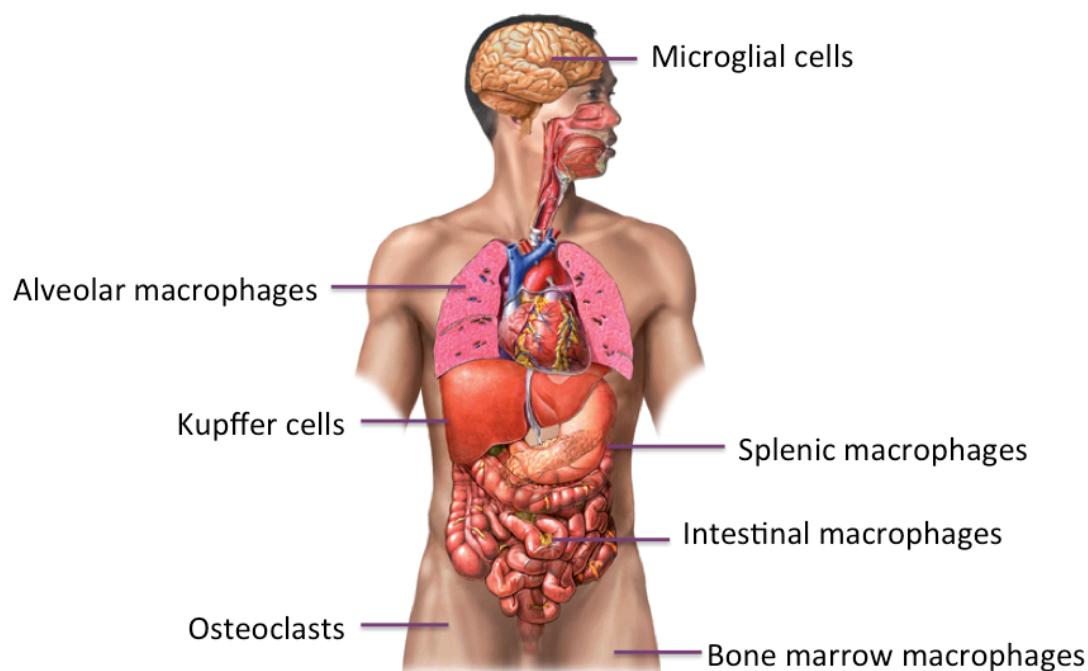


Figure 1. Different macrophage populations in the body (picture of the human body adapted from Adam)

Macrophages are found in many different tissues in the body. In the brain they are known as microglial cells, in the liver as kupffer cells and at other sites as outlined above.

1.3.1 M1 macrophages (classically activated)

M1 macrophages are associated with infections such as *Listeria monocytogenes*,⁴⁷ *Salmonella typhimurium*⁴⁸, *Escherichia coli*,⁴⁹ *Streptococcus sp.*,⁵⁰ early *Mycobacterium tuberculosis*,⁵¹ *Mycobacterium ulcerans*⁵² and *Mycobacterium avium*.⁵³ Animal experiments have shown that in *Mycobacterium tuberculosis* infection M1 macrophage induction is critical to the control of infection, whereas M2 macrophage polarisation supports intracellular persistence of the bacteria.⁵⁴

Pro-inflammatory cytokines such as TNF- α , IL-6, IL-12, induced by M1 macrophages are functional small protein molecules with low molecular weights.⁵⁵ They are produced in a sequential fashion in response to triggering of the innate immune system by invading pathogens⁵⁶. The M1 phenotype is characterised by high levels of these pro-inflammatory cytokines, the release of superoxide species and the promotion of a Th1 response⁵⁷. M1 macrophages also display high phagocytic and bactericidal potential. Inducible nitric oxide (iNOS) which causes the breakdown of arginine to citrulline and nitric oxide, plays a key role in the killing of intracellular pathogens and is upregulated in M1 macrophages⁵⁸.

1.3.2 Signaling in M1 macrophages

M1 macrophage polarisation is induced by activation of IRF/STAT signalling pathways by cytokines, such as IFNs and/or microbial products or LPS through recognition by the TLRs. STAT-mediated activation of macrophages is mediated by members of the SOCS family. M1

macrophage polarisation was originally reported to require both IFN- γ and TNF- α ; however, TLR ligands activate the MyD88-dependent pathway,⁵⁹ resulting in the generation of IFN- β .⁶⁰ This IFN- β can cause activation of classically activated macrophages. M1 cells are reported to have an IL-12^{high} IL23^{high} IL-10^{low} phenotype.

The functions of IFN- γ include enhanced microbial killing, increased antigen presentation and enhanced inflammatory cytokine production.⁶¹ IFN- γ signals through the JAK-STAT pathway when activating of macrophages, stimulating STAT1 predominantly. Mice lacking IFN- γ are more susceptible to infection with a variety of microorganisms including various bacterial, protozoal and viral infections.^{62,63} M1 macrophages have been induced *in vitro* using a combination of IFN- γ (1×10^3 U/ml recombinant human IFN- γ for 48 hours) and LPS (10 ng/ml for the last 24 hours of culture).⁶⁴ IFN- γ 's prime macrophages for prolonged and sustained expression of pro-inflammatory cytokine genes, in response to PAMPs. IFN- γ is a very potent inducer of the M1 polarization state⁶⁵ and interestingly, prevents tolerance by preserving the expression of receptor-interacting protein 140 (RIP140) co-activator and promoting TLR-induced chromatin accessibility upon secondary TLR challenge.^{66,67} In addition to M1 or M2 activation states, macrophages can also enter a tolerant state. This tolerant state occurs whereby during either endotoxin shock or acute sepsis, a pro inflammatory cytokine response is induced, but importantly, on second endotoxin challenge macrophages can become hypo-responsive.⁶⁸ As a result, pro-inflammatory responses are downregulated reducing collateral inflammatory damage.

TNF- α and IL-1 are two of the most well studied pro-inflammatory cytokines. TNF- α is a 17 kDa protein that is released from macrophages within 30 minutes of the onset of a stimulus such as inflammation or invasive infection. TNF- α enhances the production of macrophages

from progenitor cells⁶⁹ and also encourages activation and differentiation of macrophages⁷⁰ as well as prolonged survival.⁷¹ IL-1 is also released predominantly by macrophages. Both cytokines act together to induce a shock-like state which is characterised by vascular permeability and haemorrhage.⁷²

Numerous studies suggest that M1 polarisation affords protection during acute infections.⁷³ However when the M1 polarisation profile loses the normal homeostatic mechanisms then an exaggerated response is detrimental to the host, for example: in a baboon model of peritonitis, animals with a prominent M1 phenotype were more likely to die compared with those who had a mixed M1/M2 macrophage polarisation profile.⁷⁴

Table 5. Markers associated with M1 and M2 polarisation states

M1 macrophages	M2 macrophages
High oxygen consumption	Preferentially express receptors for foreign antigens
Phagocytose intracellular pathogens	
Cytotoxicity	Produces arginase, IL-Ra, IL-10
Express iNOS	
Secrete: nitric oxide pro-inflammatory cytokines (IL-1, IL-6, TNF- α) Th1 response associated cytokines (IFN- γ , IL-12, IL-18)	Produce CCL17, CCL22

Some differences exist between markers of M1 and M2 polarisation states in mice and humans and these are outlined in Table 6. Overall, the Th1 and Th2 responses are similar between the species, as well as the cytokine response.

Table 6. Differences between M1 and M2 markers in humans and mice

Properties	Human		Mouse	
	M1	M2	M1	M2
Fc and scavenger receptors	FcγRI, FcγRII, FcγRIII	FcεRII, mannose receptor Scavenger receptor B-glucan receptor		Mannose receptor
Chemokine receptors	CCR7	CCR2, CXCR1, CXCR2		
Bactericidal activity	Yes	No	Yes	No
Inhibition of IFNγ production	No	Yes		
Th1/Th2 polarisation	Th1 response	Th2 response	Th1 response	Th2 response
Nitric oxide	Yes – production of iNOS	No – production of arginase	Yes – production of iNOS	No – production of arginase
Cytokines	TNFα, IL-12, IFNα/β, IFNγ	IL-1Ra, IL-10	TNFα, IL-12, IFNα/β, IFNγ	IL-1Ra, IL-10
Chemokines	CCL3, CCL4, CCL5, CXCL9, CXCL10	CCL16, CCL17, CCL18, CCL22, CCL24	CCL3, CCL5	CCL17
Specific molecules		βIG-H3		FIZZ1/RELMα

1.3.3 M2 macrophages

M2 macrophages are involved in the resolution of inflammation and tissue repair, as well as angiogenesis and tumour progression. In the 1990's, a role for IL-4 in M2 macrophage polarisation was described.⁷⁵ Polarisation of macrophages to the M2 profile is also induced by IL-13. IL-4 and IL-13 are both well-known activators of alternative macrophage phenotypes,⁷⁶ as well as being associated with parasitic infections. Other cytokines such as IL-33 and IL-25 can amplify M2 polarisation indirectly.⁷⁷ Alternatively activated macrophages have well-described anti-inflammatory effects and have been characterised by high levels of TGF- β , IL-10, M-CSF. M2 markers also include arginase-1 (Arg-1), mannose receptor (MR), chitinase 3-like 3 (Ym-1) and resistin-like molecule- α (RELM- α , also known as Fizz-1). Other markers associated with this phenotype are IFN regulatory factor 5 (IRF-5), Kruppel-like factor 4 (KLF-4), suppressor of cytokine signaling 1 (SOCS-1) and transglutaminase 2 (TGM2).⁷⁸ M2 macrophages convert arginine to ornithine and urea through the action of Arg-1.⁷⁹ Studies with IL-4R knockdown mice showed that they were highly susceptible to infection with *Schistosoma mansoni*, with mortality attributed to an M1-driven cytokine response and elevated iNOS activity.⁸⁰ IL-4 and IL-13 are associated with a Th2 type immune response, which is involved in the immune response to allergens and parasites. IL-4 has a distinct function in skewing macrophage polarisation and is needed for efficient phagocytosis. An M1 to M2 switch can occur during the change from acute to chronic infection and allows for protection from overwhelming inflammation, thus the M2 polarization profile is linked with the persistence of pathogenic bacteria in tissues and the chronicity of infectious diseases. Once such bacterium which exploits this chronic M2 profile, is mycobacterium.⁸¹

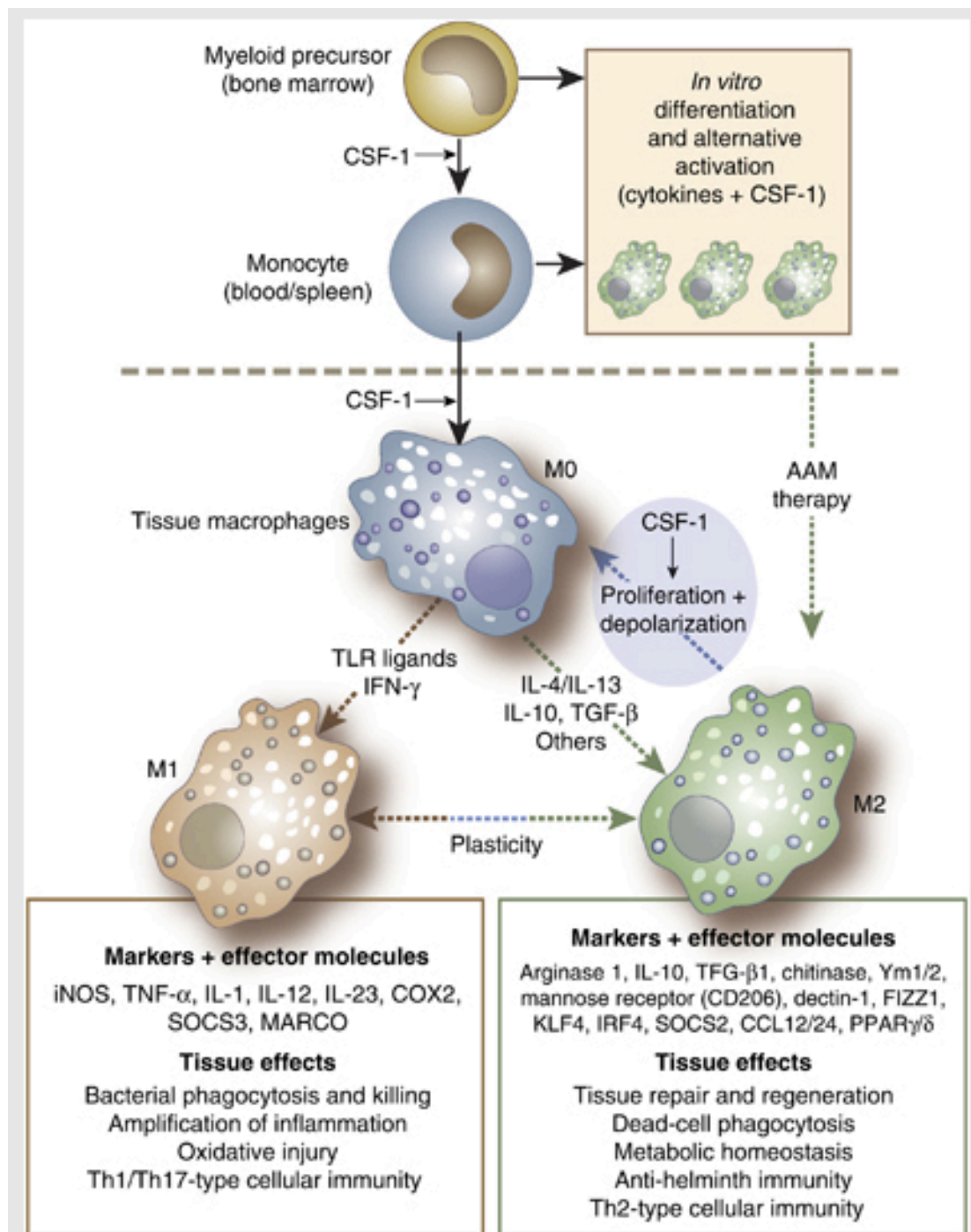


Figure 2. M1 and M2 macrophages and their phenotypic differences

M1 and M2 macrophages have varying phenotypic functions depending on their polarisation state. Highlighted above are the different polarising stimuli, characteristic markers, cytokine profiles and functions of the M1 and M2 polarisation states.

1.3.4 Role of macrophages in the immune system

Macrophages are potent phagocytic cells that are involved in the clearance of cellular debris and cells that have undergone apoptosis. They are rapidly recruited to wounds following platelet degranulation.⁸² They detect endogenous danger signals through the TLR family, other PRRs and IL-1R. The majority of these pathways signal through the adaptor protein MyD88. Mosser and Edwards suggest three classifying categories for macrophages according to their functions: host defence, wound healing and immune response.⁷⁶

It is well established that macrophages display extraordinary plasticity with the ability to switch between phenotypes *in vitro* and *in vivo*.^{83 84} Macrophages display plasticity resulting in a spectrum of macrophage activation dependent on environmental signals.⁸⁵ Phenotypic switches can occur in macrophage populations over time, but it is unclear whether this switch is due to switching of the macrophage phenotype back to the resting state, or whether it is due to infiltration of tissues with new populations of macrophages.⁷⁶ Macrophages also exhibit numerous cell surface markers, which enable researchers to divide them into subpopulations based on their phenotypic functions. Early warning signs can trigger macrophage activation and allow for macrophage recruitment and *in situ* activation and proliferation.⁸⁶ The sensing of tissue damage enables further macrophage activation, allowing for the orchestration of the host immune defense. Following this the production of anti-inflammatory signals, culminates either, in the re-establishment of homeostasis, or chronic infection or inflammation.

1.3.5 Macrophage polarisation and its role in sepsis

The term macrophage polarisation was first used by Mackaness in the 1960's.⁸⁷ Activation of macrophages has emerged as a key area of research. Macrophages are involved in a wide variety of immune processes including immunology, tissue homeostasis, disease modulation and resolution of inflammation. Macrophage activation can be influenced by a variety of factors including cytokines, pathogens and endotoxins. Mills and colleagues suggested that macrophage polarisation be split into two categories as defined by the ability of M1 macrophages to secrete NO and M2 macrophages to make trophic polyamines.⁸⁸ M1 macrophages are associated with a profound pro-inflammatory state, whereas M2 macrophages are associated with an anti-inflammatory state. These opposing effects are vital for regulation of the inflammatory response. The massive pro-inflammatory response that is associated with bacterial infection must be tempered with a variety of regulatory anti-inflammatory mechanisms in order to avoid a deleterious overwhelming inflammatory response. The main mode of death in septic patients is multi-organ failure as a result of damage from the overwhelming release of pro-inflammatory cytokines. Macrophage polarisation is driven by signals in the microenvironment, which shapes the phenotype of the activated macrophages.

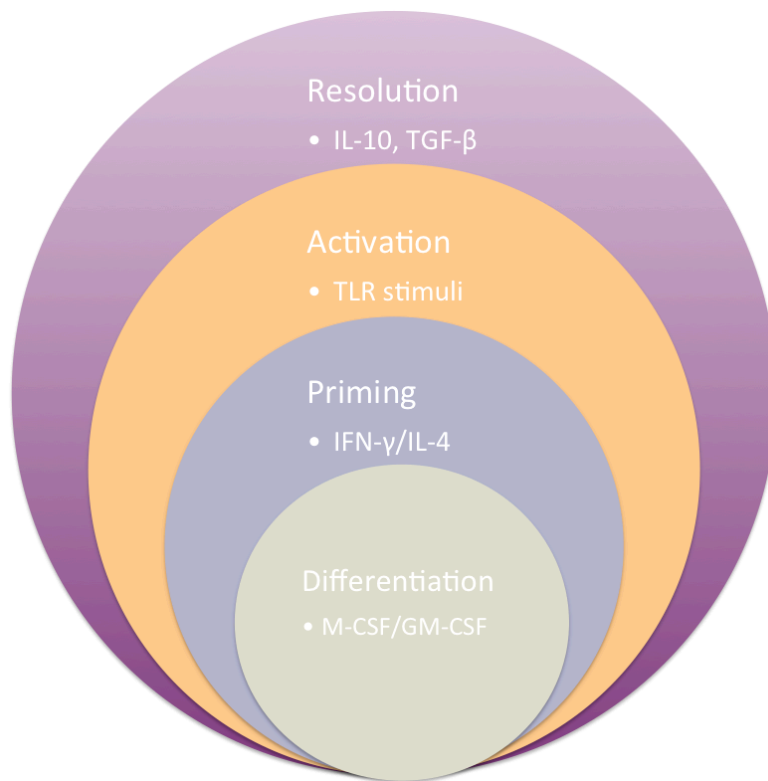


Figure 3. Paradigm of macrophage activation (adapted from ⁸⁹)

There are different stages in the macrophage activation paradigm. Monocytes mature into macrophages in the presence of M-CSF and GM-CSF. Following this they can be primed by different stimuli including M-CSF and GM-CSF. Macrophages are polarised towards phenotypes by substances such as IL-4 and IFN- γ . M2 macrophages are involved in the resolution of inflammation and in tissue repair and IL-10 and TGF- β are the characteristic cytokines associated with this stage.

1.4 Signalling pathways involved in Sepsis

1.4.1 Toll-like receptors

The transmembrane TLRs with an extracellular domain involved in bacterial ligand recognition are the most widely described PRRs. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are located at the extracellular surface, whereas TLR3, TLR7, TLR8 and TLR9 are located in the endoplasmic reticulum and endosomes.^{90,91,92,93} TLRs were initially investigated in *Drosophila*⁹⁴, which has no adaptive immune system.⁹⁵ Eleven TLRs have been discovered in humans and thirteen in mice⁹⁶ and they or their homologues are found in all multi-cellular organisms.⁹⁷ Species differences do occur in the TLRs, which complicates attempts at cross-species direct comparisons. TLR2 and TLR4, which are expressed on the cell surface, are perhaps the most widely investigated of the TLR family and the only TLRs shown to be responsive to microbial ligands.⁹⁸ LPS or endotoxin, derived from gram-negative bacteria almost exclusively activates its primary receptor TLR4, one of the most studied pathways in host innate immunity against gram-negative bacterial infection.

1.4.2 TLR4

TLR4, initially named hToll was discovered in the 1990's, when Hoshino and colleagues, using TLR4-deficient mice, demonstrated the hypo-responsiveness of these animals to LPS stimulation,⁹⁹ thus confirming the pivotal role of TLR4 in the response to LPS. TLR4-deficient mice have been shown to be susceptible to gram-negative bacterial infection. In addition, specially bred mice that exclusively expressed TLR4 on endothelial cells were found to be more efficient at clearing *Escherichia coli* infection.¹⁰⁰ Smirnova *et al*, on examining DNA from patients with meningococcal disease found that a variant in the TLR4

gene is associated with an increased susceptibility to meningococcal septicaemia.¹⁰¹ Genetic variants in the TLR4 have also been linked to gram-negative bacterial infection in neonates.¹⁰²

1.4.3 TLR2

TLR2, on the other hand, forms heterodimers TLR2/TLR1 and TLR2/TLR6 with either TLR1 or TLR6, and is a functional receptor for components of gram-positive bacteria including LTA, peptidoglycan (PGN) and bacterial lipopeptides, thus being responsible for the detection of gram-positive bacteria.^{103,104,105} TLR2-deficient mice are highly susceptible to gram-negative *Staphylococcus aureus* infection, with significantly attenuated TNF- α and IL-6 production.¹⁰⁶ TLR5 and TLR9 recognize flagellin of bacteria flagella and bacterial CpG-DNA,^{107,108} respectively.

1.4.4 TLR signaling in sepsis

Upon engagement with their specific ligands, TLRs activate several intracellular signalling pathways. Signalling by TLRs in humans involves a family of five adaptor proteins, which interact with downstream protein kinases that ultimately lead to the activation of transcription factors including nuclear factor- κ B (NF- κ B) and members of the interferon (IFN)-regulatory factor (IRF) family. The Toll/interleukin-1 (IL-1) receptor (TIR) domain, which is unique to the TLR system, is the key signalling domain for not only TLRs but also the adaptor protein. These five adaptor proteins include myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (MAL), TIR-domain-containing adaptor protein inducing IFN- β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile- α and armadillo-motif-containing protein (SARM).¹⁰⁹ MAL, TRIF and TRAM are also known as TIR domain-containing adaptor

protein (TIRAP), TIR-containing adaptor molecule-1 (TICAM1) and TICAM2. All TLRs (except TLR3) activate the MyD88 pathway, which results in the activation predominantly of the downstream NF- κ B and mitogen-activated protein kinase (MAPK) signalling pathways, and ultimately leads to the production of inflammatory cytokines.

1.4.5 MyD88 dependent pathway

Upon stimulation, MyD88 recruits IL-1 receptor-associated kinase (IRAK) family to TLRs, and IRAK1 then associates with TNF receptor-associated factor 6 (TRAF6). This subsequently leads to the activation of NF- κ B as well as MAPKs including p38, c-Jun NH2-terminal kinase (JNK) and extracellular signal-related kinase 1/2 (ERK1/2).¹¹⁰ Macrophages and DCs isolated from MyD88-deficient mice have been shown to be unable to respond to certain TLR ligands including TLR2, TLR5, TLR7 and TLR9,¹¹¹ indicating that these TLRs are fully dependent on the MyD88 signalling in order to activate the NF- κ B signalling pathway. These cells however can remain somewhat responsive to LPS stimulation through a MyD88-independent pathway. TLR3 and TLR4 can activate a MyD88-independent/TRIF-dependent pathway, which allows for the activation of NF- κ B and IRF3, and induction of IFN- β . TRIF (also known as TICAM1) activates TRAF3 and TRAF6, and signalling from TRAF3 induces IRF3 activation and allows for the production of IFN- β . Mice lacking TRIF fail to generate a type I IFN response to LPS stimulation, though their ability to activate the NF- κ B and MAPK signalling pathways is preserved.¹¹² TRAM links TRIF to TLR4, and studies have shown TLR4 to possess the most complex signalling mechanism of all the TLRs, as TLR4 is the only member of the TLR family that recruits four adaptor proteins MyD88, MAL, TRIF and TRAM and activates two signalling pathways, namely the MyD88- and TRIF-dependent pathways.¹¹³

1.4.6 p38 MAPK signaling in sepsis

The p38 family is a major player in the host inflammatory response, particularly in macrophages. It is activated in response to a variety of stimuli, including pathogens, cytokines, growth factors and UV radiation¹¹⁴. P38 signalling varies depending on the stimulus. p38 has four distinct isoforms; α , β , γ and δ . The expression of inflammatory mediators, e.g. IL-1 β , TNF- α , PGE2, IL-12, COX-2, IL-8, IL-6, IL-3, IL-2 and IL-1 on macrophages is mediated by p38 α . p38 allows for the binding of NF- κ B to targets on IL-8 and MCP-1. Endotoxin, TNF- α , platelet aggregating factor (PAF) and IL-1 induce p38 in innate immune cells, which is an essential step for the release of inflammatory cytokines and chemokines, though when there is prolonged activation of p38 a hypimmune state can occur, which is associated with the latter stages of sepsis. Recent studies have described an impaired pro-inflammatory response of macrophages from septic patients to *in vitro* stimulation with CD40L, while survival is associated with the recovery of a pro-inflammatory response¹¹⁵. This impaired response is related to the antigen presenting capacity of macrophages in septic patients which becomes impaired by 24 hours and recovers only after up to 14 days.¹¹⁶ In survivors of sepsis other studies demonstrated 19 of 14,500 genes were overexpressed and these were mainly involved in the innate immune response.¹¹⁷

MAPKs are Ser-Thr kinases, which activate a number of transcription factors. There are three main MAPK pathways; the ERK1/2 pathway, p38 pathway and JNK pathway. The MAPK pathways are activated by a number of phosphorylation events beginning with phosphorylation of the MAPK kinases at 2 serine residues by MAPK kinase kinases (MKKs). These activated MAPK kinases phosphorylate MAPKs at the threonine and tyrosine residues.

Activated MAPKs phosphorylate a wide variety of downstream molecules including protein kinases and transcription factors. MAPKs can affect the transcriptional regulation of mRNAs, thereby modifying their stability, transport and translation.¹¹⁸ These signalling cascades are involved in many normal cellular functions but are also activated in a variety of pathologies including septic shock.¹¹⁹ The MAPK signalling pathways mediates the release of a number of different inflammatory cytokines in cells exposed to bacterial stimulation.¹²⁰ Bone marrow-derived macrophages (BMDMs) grow in response to a number of different stimuli including colony-stimulating factor-1 (CSF-1) or macrophage colony-stimulating factor (M-CSF), which allows for progenitor cells to mature into monocytes and macrophages and for their survival and activation into mature macrophages. In BMDMs, stimulation with M-CSF activates the MAPK signaling pathway.¹²¹

In addition to the above, MAPK is involved in the regulation of inducible nitric oxide synthase (iNOS), which is an important marker of M1 polarisation. Nitric oxide has a number of important functions in the immune response; it is involved in tumour cell death, killing of intracellular pathogens, vasodilatation, inhibition of platelet aggregation and neurotransmission.¹²² There are three forms of NOS; endothelial, neuronal and inducible. Increased expression of iNOS is associated with sepsis as well as other conditions such as haemorrhagic shock, rheumatoid arthritis and tuberculosis.

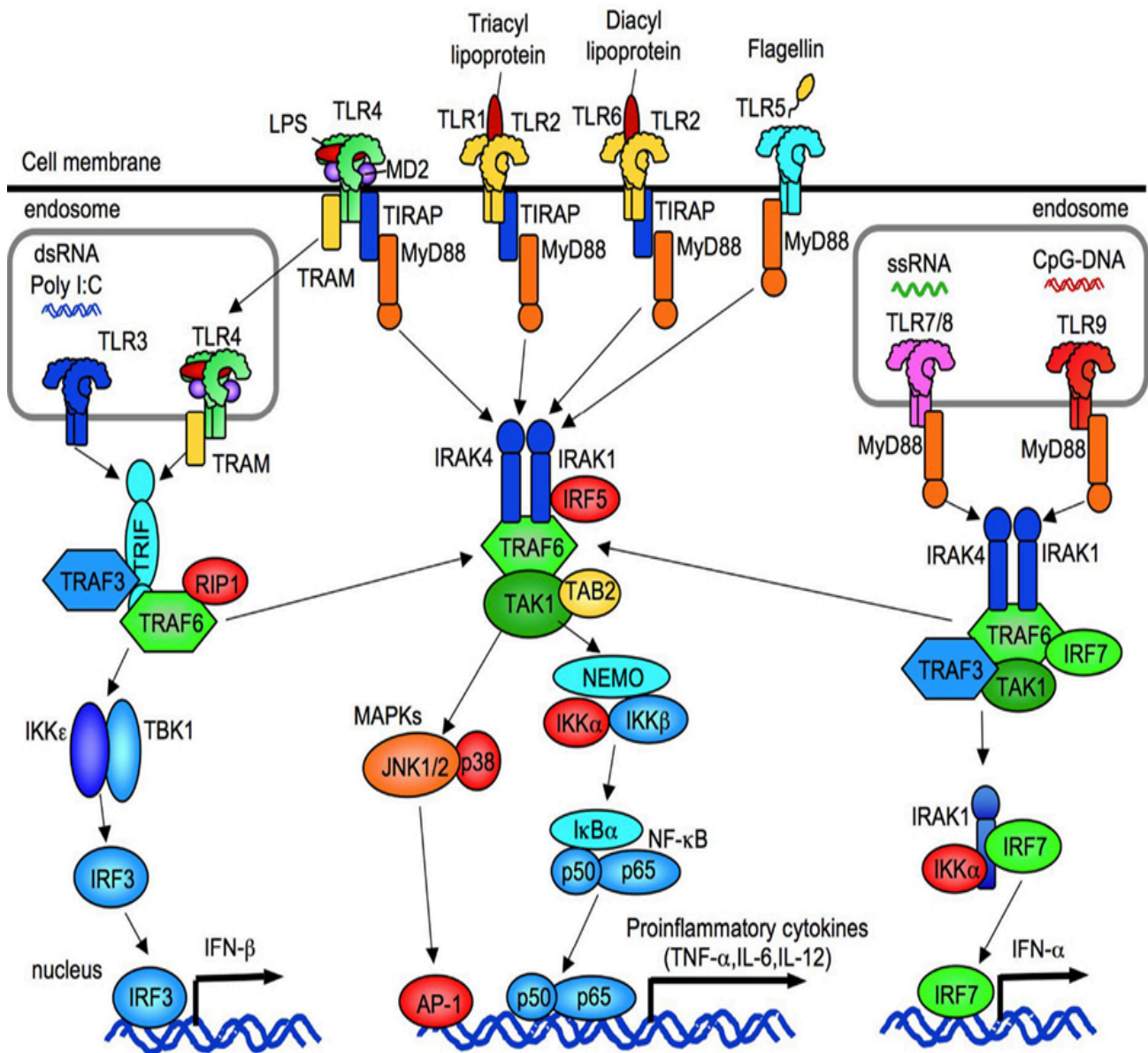


Figure 4. Schematic representation of TLR, MAPK and NF-κB signalling in sepsis

1.5 Endotoxin tolerance

The host response to microbial infection involves a period of massive inflammatory cytokine production. This is then followed by a period known as endotoxin tolerance, when the host becomes hyporesponsive to stimulation with LPS. This was first described in animals that were injected with a sub-lethal dose of bacterial endotoxin and followed by a fatal dose. Paul Beeson reported on endotoxin tolerance in 1946 when he described the abrogation of fever in rabbits undergoing repeated injections of typhoid vaccine.¹²³ In animal models, two phases of endotoxin tolerance have been outlined: an early phase characterised by altered cellular activation and a later phase associated with the development of specific antibodies against the polysaccharide side chain of gram-negative organisms.¹²⁴ Monocytes and macrophages exposed to endotoxin for between 3 and 24 hours became tolerant and display an altered response to re-challenge with bacterial endotoxin or lipopolysaccharide. One study showed that treatment of human monocytes with LPS for even one hour could induce an endotoxin tolerant state.¹²⁵ This is important because there is a multitude of evidence supporting the fact that immune cells, mainly monocytes and macrophages, from patients with sepsis display many of the characteristics of endotoxin tolerance.¹²⁶

1.5.1 The role of endotoxin tolerance in sepsis

Endotoxin tolerance is associated with protection against tissue damage and mortality in animal models of sepsis. It is not an anti-inflammatory state, but more a cellular reprogramming leading to immune hypo-responsiveness.¹²⁷ The association between endotoxin tolerance and sepsis is very strong. Circulating monocytes isolated from patients with sepsis have shown similar characteristics to that seen in endotoxin tolerance.¹²⁵ *In vitro* and *in vivo* models of endotoxin tolerance exist, classically where cells are stimulated twice

with LPS. Initially a low or sub-lethal dose of LPS is administered which is followed by administration of a higher dose. For example, when peripheral blood mononuclear cells are isolated from septic patients and exposed to LPS or other TLR4 ligands they are shown to develop a hypo-responsiveness to a second stimulus. The period of hypo-responsiveness to a second dose of LPS is time-dependent and previous work has demonstrated that cells regain the ability to mount a pro-inflammatory response after 5 days.¹²⁸

1.5.2 Similarities between endotoxin tolerance and the M2 macrophage polarisation profile

Many of the characteristics of endotoxin tolerance resemble the immunosuppressive M2 macrophage phenotype. Endotoxin tolerant monocytes are however, different to the M2 macrophage polarisation phenotype, which is dependent on the context, stimulus and method of tolerisation. In murine models of endotoxin tolerance, IL-6 and IFN- γ released following LPS challenge are dramatically reduced but IL-12p70 is not as significantly reduced.¹²⁹ TNF- α is the best marker of endotoxin tolerance, because of its significantly reduced production in tolerised cells.¹³⁰ The suppressed production of pro-inflammatory cytokines is as a result of alterations in the NF- κ B and MAPK signalling pathways. Previous research has demonstrated that pretreatment of macrophages with IL-4 does not reduce the LPS-induced expression of pro-inflammatory genes, MAPK activation or NF- κ B binding.¹³¹

Interestingly, it is well established that IFN- γ rescues monocytes and macrophages from endotoxin tolerance.^{132,133,126} In the absence of IFN- γ or GM-MCSF, TLR or TNF- α induces only a brief M1 activation state, which quickly becomes a more tolerant M2-like state.¹³⁴

NF- κ B is essential for the optimum production of pro-inflammatory cytokines in inflammation. Tolerance is associated with impaired NF- κ B activation and a reduction in the p65 and p50 heterodimer binding and pro inflammatory gene transcription. The production of IFN- β which polarizes macrophages to an M1 state is suppressed by p50 NF- κ B inhibition of NF- κ B signalling.⁶⁸ Studies have shown a reduced level of NF- κ B in survivors of sepsis, while non-survivors had a prominent inactive homodimer compared to controls.¹³⁵ Following stimulation with LPS, the peripheral blood mononuclear cells of septic patients produce lower levels of NF- κ B, similar to those seen during tolerisation.

Transcriptional profiling in macrophages has revealed two sets of LPS responsiveness genes: those that are tolerizable, e.g. TNF- α and IL-6, and those that are non-tolerizable, e.g. antimicrobial and anti-inflammatory genes. Chromatin changes are associated with LPS tolerance and it is postulated that this transcriptional signature drives a phenotypic switch in macrophage polarisation, from a pro-inflammatory to an anti-inflammatory phenotype.¹²⁷ LPS tolerance switches macrophages to an anti-inflammatory phenotype which is distinct from an M2 phenotype.¹³¹ Alternatively, activated macrophages induced by IL-4, retain the ability to respond to TLR ligands and can induce pro-inflammatory cytokines.¹³¹ Recovery from LPS tolerance may allow macrophages to mount an efficient immune response, while also protecting against over-activation of the inflammatory response.

1.6 Summary

Through a variety of signaling pathways, invading pathogens are sensed through pathogen recognition receptors and an immune response is mounted. When acting appropriately this response will result in the elimination of the bacteria and resolution of inflammation with the minimum of tissue damage. The immune response can, however, deviate from its normal homeostatic mechanisms and result in massive tissue damage, end organ damage and multi-organ failure from an overwhelming immune response. Macrophages are the backbone of the immune response, from their role in detecting invading pathogens through PRRs, to the activation of signaling pathways and the release of pro-inflammatory cytokines. The two main macrophage polarisation states, M1 and M2 have varying functions from mounting the acute cytokine response to their activity in tissue remodelling and repair. Being able to manipulate or direct the immune response is potentially very useful, from switching off the overwhelmingly M1 reaction to switching M2 macrophages back to an M1 state during the immune hyporesponsive period that can occur.

The aim of this thesis is; To characterise the response of naïve and polarised macrophages in an *ex –vivo* model of bacterial infection.

¹ Geroulanos, S. and E. T. Douka (2006). **Historical perspective of the word "sepsis"**. *Intensive Care Med* 32(12): 2077.

² Hippocrates (1849). **Of Epidemics. The Genuine Works Of Hippocrates**. A. Francis. London, Sydenham Society: 339-381.

³ Pollak, K. (1970). **Los discípulos de Hipócrates, Una historia de la Medicina**. Barcelona, Círculo de Lectores.

⁴ Magner, L. (2009). **A History of Infectious Diseases and the Microbial World**. New York, Praeger Publishers.

-
- ⁵ Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, et al. **The Surviving Sepsis Campaign Guidelines Committee including the Paediatric Subgroup. Surviving Sepsis Campaign: International Guidelines for Management of Severe Sepsis and Septic Shock, 2012** *Intensive Care Med* (2013) 39:165–228
- ⁶ Martin GS, Mannino DM, Eaton S, Moss M. **The epidemiology of sepsis in the United States from 1979 through 2000.** *N Engl J Med* 2003 Apr 17;348(16):1546-54.
- ⁷ Lever A, Mackenzie I. **Sepsis: definition, epidemiology, and diagnosis.** *BMJ: British Medical Journal* 2007;335(7625):879-883
- ⁸ **American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis.** *Crit. Care Med.* 1992; 20(6):864–874. No authors listed.
- ⁹ Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsay G;SCCM/ESICM/ACCP/ATS/SIS. **2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference.** *Crit Care Med.* 2003 Apr;31(4):1250-6.
- ¹⁰ Martin GS, **Sepsis, severe sepsis and septic shock: changes in incidence, pathogens and outcomes.** *Expert Rev Anti Infect Ther.* 2012 June;10(6):701–706
- ¹¹ Hall MJ, Williams SN, DeFrances CJ, Golosinskiy A. **Inpatient care for septicemia or sepsis: a challenge for patients and hospitals.** *NCHS Data Brief.* 2011;(62):1-8.
- ¹² Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. **Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care.** *Crit Care Med* 2001 Jul;29(7):1303-10
- ¹³ Alberti C, Brun-Buisson C, Burchardi H, Martin C, Goodman S, Artigas A, Sicignano A, Palazzo M, Moreno R, Boulmé R, Lepage E, Le Gall R. **Epidemiology of sepsis and infection in ICU patients from an international multicentre cohort study.** *Intensive Care Med* Feb ;28(2):108-21. Epub 2001 Dec 4
- ¹⁴ Wang HE, Donnelly JP, Shapiro NI Hohmann SF, Levitan EB. **Hospital Variations in severe sepsis mortality.** *Am J Med Qual.* 2015 Jul-Aug;30(4):328-26
- ¹⁵ Gaieski DF, Edwards JM, Kallan MJ, Carr BG. **Benchmarking the incidence and mortality of severe sepsis in the United States.** *Crit Care Med* 2013 May;41(5):1167-74.
- ¹⁶ Chalupka AN, Talmor D. **The economics of sepsis.** *Crit Care Clin* 2012 Jan;28(1):57-76
- ¹⁷ Manns BJ, Lee H, Doig CJ, Johnson D, Donaldson C. **An economic evaluation of activated protein C treatment for severe sepsis.** *N Engl J Med* 2002 Sep 26;347(13):993-1000.

-
- ¹⁸ Angus DC, Linde-Zwirbler WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. **Epidemiology of severe sepsis in the United States: analysis of incidence, outcome and associated costs of care.** *Crit Care Med*. 2001 Jul;29(7):1303-10
- ¹⁹ Lagu T, Rothberg MB, Shieh MS, Pekow PS, Steingrub JS, Lindenauer PK. **Hospitalizations, costs, and outcomes of severe sepsis in the United States 2003 to 2007.** *Crit Care Med* 2012 Mar;40(3):754-61
- ²⁰ Beveridge TJ. **Use of the gram stain in microbiology.** *Biotech Histochem* 2001 May;76(3):111-8
- ²¹ Vincent JL, Sakr Y, Sprung CL, Ranieri VM, Reinhart K, Gerlach H, Moreno R, Carlet J, Le Gall JR, Payen D, **Sepsis Occurrence in Acutely Ill Patients Investigators.** *Crit Care Med* 2006 Feb;34(2):344-53
- ²² Vincent JL, Rello J, Marshall J, Silva E, Anzueto A, Martin CD, Moreno R, Lipman J, Gomersall C, Sakr Y, Reinhart K, **EPIC II Group of Investigators. International study of the prevalence and outcomes of infection in intensive care units.** *JAMA* 2009 Dec 2;302(21):2323-9
- ²³ Friedman G, Silva E, Vincent JL. **Has the mortality of septic shock changed with time.** *Crit Care Med* 1998 Dec;26(12):2078-86.
- ²⁴ Ibrahim EH, Sherman G, Ward S, Fraser VJ, Kollef MH. **The influence of inadequate antimicrobial treatment of bloodstream infections on patient outcomes in the ICU setting** *Chest*, 2000;118:146–55
- ²⁵ Beal AL, Cerra FB. **Multiple organ failure syndrome in the 1990s: systemic inflammatory response and organ dysfunction.** *JAMA* 1994;271:226-233
- ²⁶ Raghaven M, Marik PE. **Management of sepsis during the early “golden hours”.** *J Emerg Med* 2006 Aug;31(2):185-99
- ²⁷ Rivers E, Nguyen B, Havstad S, Ressler J, Muzzin A, Knoblich B et al. **Early goal-directed therapy in the treatment of severe sepsis and septic shock** *N Engl J Med*, 2001 Nov 8;345(19):1368-77
- ²⁸ Holder AL, Huang DT. **A dream deferred: the rise and fall of recombinant activated protein C.** *Crit Care* 2013 Mar 12;17(2):309
- ²⁹ Sha T, Iizawa Y, Li M. **Combination of Imipenem and Tak-242, a Toll-like receptor 4 signal transduction inhibitor, improves survival in a murine model of polymicrobial**

sepsis. *Shock*. 2011 Feb;35(2):205–9.

³⁰ Takeda [http://www.takeda.com/news/2009/20090220_3678.html]

³¹ Rice T, Wheeler A, Bernard G, Vincent JL, Angus D, Aikawa N, et al. **A randomized, double-blind, placebo-controlled trial of TAK-242 for the treatment of severe sepsis.** *Crit Care Med*. 2010 Aug;38(8):1685–94. ^[L]_{SEP}

³² Wang H, Ward MF, Sama ME. **Targeting HMGB1 in the treatment of sepsis.** *Expert Opinion on Therapeutic Targets* 2014; 18(3):257-268

³³ Thomas L. **Germs.** *N Engl J Med* 1972; 287(11):553–555

³⁴ Martin GS, Mannino DM, Moss M. **The effect of age on the development and outcome of adult sepsis.** *Crit Care Med* 2006 Jan;34(1):15–21

³⁵ Biron CA. **More things in heaven and earth: defining innate and adaptive immunity.** *Nat Immunol*. 2010 Dec;11(12):1080-2

³⁶ Molecular Biology of the Cell. 4th Ed. Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P.

³⁷ Wiersinga WJ, Leopold SJ, Cranendonk DR, van der Poll T. **Host innate immune response to sepsis.** *Virulence*. 2014;5(1):36-44

³⁸ Foley NM, Wang J, Redmond HP, Wang JH. **Current knowledge and future directions of TLR and NOD signaling in sepsis.** *Mil Med Res* 2015;2:1

³⁹ Newton K, Dixit VM. **Signaling in innate immunity and inflammation.** *Cold Spring Harb Perspect Biol*. 2012;4(3);a006049

⁴⁰ Takeuchi O, Akira S. **Pattern recognition receptors and inflammation.** *Cell* 2010 Mar 19;140(6):805-20

⁴¹ Geissmann, F. Manz MG, Jung S, Sieweke MH, Merad M, Ley K. **Development of monocytes, macrophages, and dendritic cells.** *Science* 2010 Feb 5;327(5966):656–61

⁴² Gordon S, Taylor PR. **Monocyte and macrophage heterogeneity.** *Nature Rev Immunol*. 2005 Dec;5(12):953–64

⁴³ Ginhoux F, Jung S. **Monocytes and macrophages: developmental pathways and tissue homeostasis.** *Nat Rev Immunol* 2014 14;392-404

⁴⁴ Metchnikoff E. **Leçons sur la pathologie comparée de l'inflammation** (Masson, 1892).

-
- ⁴⁵ Ajuebor MN, Das AM, Virag L, Flower RJ, Szabo C, Perretti M. **Role of resident peritoneal macrophages and mast cells in chemokine production and neutrophil migration in acute inflammation: evidence for an inhibitory loop involving endogenous IL-10.** *J. Immunol.* 1999 Feb 1;162(3):1685–91
- ⁴⁶ Rosas M, Liddiard K, Kimberg M, Faro-Trindade I, McDonald JU, Williams DL et al. **The induction of inflammation by dectin-1 in vivo is dependent on myeloid cell programming and the progression of phagocytosis.** *J. Immunol.* 2008 Sep 1;181(5):3549–57
- ⁴⁷ Shaughnessy LM, Swanson JA. **The role of the activated macrophage in clearing *Listeria monocytogenes* infection.** *Front Biosci.* 2010;12:2683–2692.
- ⁴⁸ Jouanguy E, Doffinger R, Dupuis S, Pallier A, Altare F, Casanova JL. **IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men.** *Curr Opin Immunol.* 1999;11(3):346–351.
- ⁴⁹ Liang MD, Bagchi A, Warren HS, Tehan MM, Trigilio JA, Beasley-Topliffe LK, et al **Bacterial peptidoglycan-associated lipoprotein: a naturally occurring Toll-like receptor 2 agonist that is shed into serum and has synergy with lipopolysaccharide.** *J Infect Dis* 2005 Mar 15;191(6):939–48.
- ⁵⁰ Kadioglu, A, Andrew PW. **The innate immune response to pneumococcal lung infection: the untold story.** *Trends Immunol.* 2004 Mar;25(3):143–149.
- ⁵¹ Ehrt S, Schnappinger D, Bekiranov S, Drenkow J, Shi S, Gingeras TR et al. **Reprogramming of the macrophage transcriptome in response to interferon-gamma and *Mycobacterium tuberculosis*: signaling roles of nitric oxide synthase-2 and phagocyte oxidase.** *J Exp Med* 2001 Oct 15;194(8):1123–40.
- ⁵² Kiszewski AE., Becerril E, Aguilar LD, Kader LT, Myers W, Portaels F, Hernandez Pando R. 2006. **The local immune response in ulcerative lesions of Buruli disease.** *Clin. Exp. Immunol.* 2006 Mar;143(3):445– 51.
- ⁵³ Murphy JT, Sommer S, Kabara EA, Verman N, Kuelbs MA, Saama P, Halgren R, Coussens PM. **Gene expression profiling of monocyte-derived macrophages following infection with *Mycobacterium avium* subspecies *avium* and *Mycobacterium avium***

subspecies paratuberculosis. *Physiol. Genomics* 2006 Dec 13;28(1):67–75.

⁵⁴ Kahnert A, Seiler P, Stein M, Bandermann S, Hahnke K, Mollenkopf H, Kaufmann SH.

Alternative activation deprives macrophages of a coordinated defense program to *Mycobacterium tuberculosis*. *Eur J Immunol* 2006 Mar;36(3):631-47.

⁵⁵ Arango Duque G, Descoteaux A. **Macrophage cytokines: involvement in immunity and infectious diseases.** *Front Immunol.* 2014;5:491

⁵⁶ Laskin DL. **Macrophages and inflammatory mediators in chemical toxicity: a battle of forces.** *Chem Res Toxicol.* 2009;22(8):1376-1385

⁵⁷ Rath M, Müller I, Kropf P, Closs EI, Munder M. **Metabolism via Arginase or Nitric Oxide Synthase: Two Competing Arginine Pathways in Macrophages.** *Front Immunol.* 2014;5:532.

⁵⁸ Wang N, Liang H, Zen K. **Molecular mechanisms that influence the macrophage m1-m2 polarization balance.** *Front Immunol.* 2014;5:614

⁵⁹ Carpenter S, O'Neill LA. **How important are Toll-like receptors for antimicrobial responses?** *Cell Microbiol.* 2007 Aug; 9(8):1891-901.

⁶⁰ Hou B, Reizis B, DeFranco AL. **Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms.** *Immunity.* 2008 Aug 15; 29(2):272-82

⁶¹ Hu X, Chen J, Wang L, Ivashkiv LB. **Crosstalk among Jak-STAT, Toll-like receptor and ITAM dependent pathways in macrophage activation.** *J Leukoc Biol* 2007 Aug;82(2):237–43

⁶² Zhao YX, Tarkowski A. **Impact of interferon-gamma receptor deficiency on experimental *Staphylococcus aureus* septicaemia and arthritis.** *J Immunol* 1995 Dec;155(12):5736-42

-
- ⁶³ Way SS, Boczuk AC, Dominitz R, Goldberg MB. An essential role for gamma interferon in innate resistance to *Shigella flexneri* infection. *Infect Immun* 1998 Apr;66(4):1342-8
- ⁶⁴ Vogel DY, Glim JE, Stavenhagen AW, Breur M, Heijnen P, Amor S, Dijkstra CD, Beelen RH. Human macrophage polarization in vitro: maturation and activation methods compared. *Immunobiology*. 2014 Sep;219(9):695-703. doi: 10.1016/j.imbio.2014.05.002
- ⁶⁵ Ivashkiv LB. **Epigenetic regulation of macrophage polarization and function.** *Trends Immunol* 2013 May;34(5):216-23
- ⁶⁶ Chen J, Ivashkiv LB. **IFN-gamma abrogates endotoxin tolerance by facilitating Toll-like receptor-induced chromatin remodelling** *Proc Natl Acad Sci U S A* 2010 Nov 9;107(45):19438–43
- ⁶⁷ Ho PC, Tsui YC, Feng X, Greaves DR, Wei LN. **NF-kappaB-mediated degradation of the coactivator RIP140 regulates inflammatory responses and contributes to endotoxin tolerance** *Nat. Immunol* 2012 Mar 4;13(4):379–86
- ⁶⁸ Porta C, Rimoldi M, Raes G, Brys L, Ghezzi P, Di Liberto D et al., **Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB.** *Proc Natl Acad Sci USA* 2009 Sep 1;106(35):14978-14983
- ⁶⁹ C. Fahlman, F. W. Jacobsen, O. P. Veiby, I. K. McNiece, H. K. Blomhoff, and S. E. W. Jacobsen. **Tumor necrosis factor- α (TNF- α) potently enhances in vitro macrophage production from primitive murine hematopoietic progenitor cells in combination with stem cell factor and interleukin-7: novel stimulatory role of p55 TNF receptors.** *Blood*, 1994 84(5):1528–33
- ⁷⁰ Witsell AL and Schook LB, **Tumor necrosis factor alpha is an autocrine growth regulator during macrophage differentiation** *Proc Natl Acad Sci USA* 1992 89(10):4754–58
- ⁷¹ Conte D, Holcik M, Lefebvre CA, Lacasse E, Picketts DJ, Wright KE, et al., **Inhibitor of apoptosis protein cIAP2 is essential for lipopolysaccharide-induced macrophage survival.** *Mol Cell Biol* 2006 Jan;26(2):699-708
- ⁷² Okusawa S, Gelfand JA, Ikejima T, Connolly RJ, Dinarello CA. **Interleukin 1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition.** *J Clin Invest* 1988 Apr;81(4):1162–72

-
- ⁷³ Benoit M, Desnues B, Mege JL. **Macrophage polarization in Bacterial Infections.** *J Immunol* 2008;181:3733-3739
- ⁷⁴ Mehta A, Brewington R, Chatterji M, Zoubine M, Kinasewitz GT, Peer GT, et al **Infection-induced modulation of M1 and M2 phenotypes in circulating monocytes: role in immune monitoring and early prognosis of sepsis.** *Shock* 2004 Nov;22(5):423– 30.
- ⁷⁵ Stein M, Keshaw S, Harris N, Gordon S. **Interleukin 4 potently enhances murine macrophages mannose receptor activity: a marker of alternative immunologic macrophage activation.** *J Exp Med* 1992 Jul 1;176(1):287–92.
- ⁷⁶ Mosser DM, Edwards JP. **Exploring the full spectrum of macrophage activation.** *Nat Rev Immunol* 2008 Dec;8(2):958–69.
- ⁷⁷ O'Shea JJ, Paul WE. **Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells** *Science*, 2010;327:1098–1102
- ⁷⁸ Martinez FO, Sica A, Mantovani A, Locati M, **Macrophage activation and polarization.** *Front Biosci.* 2008;13:453-461.
- ⁷⁹ Gordon S. **Alternative activation of macrophages.** *Nat Rev Immunol* 2003. 3: 23-35
- ⁸⁰ Herbert DR, Hölscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M, et al. **Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology.** *Immunity.* 2004 May; 20(5):623-35.
- ⁸¹ Kiszewski, AE, Becerril E, Aguilar LD, Kader IT, Myers W, Portaels F, Hernandez Pando R. 2006. **The local immune response in ulcerative lesions of Buruli disease.** *Clin. Exp. Immunol.* 2006 Mar;143(3):445-51
- ⁸² Wynn TA and Barron L. **Macrophages: master regulators of inflammation and fibrosis.** *Semin. Liver Dis.* 2010;30:245–257
- ⁸³ Hagemann T, Lawrence T, McNeish, Charles KA, Kulbe H Thompson RG et al. **“Re-educating” tumor associated macrophages by targeting NF-kappaB.** *J Exp Med.* 2008;205(6):1261–1268.
- ⁸⁴ Duluc D, Corvaisier M, Blanchard S, Catal L, Descamps P, Gamelin E et al. **Interferon-gamma reverses the immunosuppressive and protumoral properties and prevents the**

generation of human tumor-associated macrophages. *Int J Cancer*. 2009;125(2):367–373.

⁸⁵ Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J. **Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences.** *J. Immunol*. 2005 Jul 1;175(1):342-9

⁸⁶ Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, van Rooijen N *et al.* **Local macrophage proliferation, rather than recruitment from the blood, is a signature of T_H2 inflammation.** *Science* 2011 Jun 10;332(6035):1284-8

⁸⁷ Mackaness GB. **Cellular resistance to infection.** *J Exp Med*. 1962 Sep 1;116:381–406.

⁸⁸ Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. **M-1/M-2 macrophages and the Th1/Th2 paradigm.** *J Immunol*. 2000 Jun 15;164(12):6166–73

⁸⁹ Gordon S, Martinez FO, **Alternative activation of macrophages: Mechanism and functions.** *Immunity* 2010 May 28;32(5):593-604

⁹⁰ Akira S, Uematsu S, Takeuchi O. **Pathogen recognition and innate immunity.** *Cell* 2006 Feb 24;124(4):783-801

⁹¹ Kopp E, Medzhitov R. **Recognition of microbial infection by Toll-like receptors.** *Curr Opin Immunol* 2003 Aug;15(4):396-401

⁹² Modlin RL, Brightbill HD, Godowski PJ. **The toll of innate immunity on microbial pathogens.** *N Engl J Med* 1999 Jun 10;340(23):1834-5

⁹³ Barton GM, Kagan JC. **A cell biological view of Toll-like receptor function: regulation through compartmentalization.** *Nat Rev Immunol* 2009, Aug;9(8):535-42

⁹⁴ Belvin MP, Anderson KV. **A conserved signaling pathway: the Drosophila toll-dorsal pathway.** *Annu Rev Cell Dev Biol* 1996;12:393-416

⁹⁵ Janeway CA, Medzhitov R. **Lipoproteins take their toll on the host.** *Curr Biol* 1999 Dec 2;9(23):R879-R82.

⁹⁶ Beutler BA. **TLRs and innate immunity.** *Blood* 2009, **113**:1399–1407.

⁹⁷ Janeway CA Jr, Medzhitov R. **Innate immune recognition.** *Annu Rev Immunol* 2002;20:197-216

-
- ⁹⁸ Brightbill HD, Modlin RL. **Toll-like receptors: molecular mechanisms of the mammalian immune response.** *Immunology* 2000 Sep;101(1):1-10
- ⁹⁹ Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. **Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product.** *J Immunol* 1999 Apr 1;162(7):3749-52
- ¹⁰⁰ Andonegui G, Zhou H, Bullard D, Kelly MM, Mullaly SC, McDonald B, Long EM, Robbins SM, Kubes P. **Mice that exclusively express TLR4 on endothelial cells can efficiently clear a lethal systemic Gram-negative bacterial infection.** *J Clin Invest* 2009 Jul;119(7):1921-30
- ¹⁰¹ Smirnova I, Mann N, Dols A, Derkx HH, Hibberd ML, Levin M, Beutler B. **Assay of locus-specific genetic load implicates rare Toll-like receptor 4 mutations in meningococcal susceptibility.** *Proc Natl Acad Sci USA* 2003 May 13;100(10):6075-80
- ¹⁰² Sampath V, Mulrooney NP, Garland JS, He J, Patel AL, Cohen JD, Simpson PM, Hines RN. **Toll-like receptor genetic variants are associated with gram-negative infections in VLBW infants.** *J Perinatol* 2013 Oct;33(10):772-7
- ¹⁰³ Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, Klimpel GR, Godowski P, Zychlinsky A. **Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2.** *Science* 1999 Jul 30;285(5428):736-9.
- ¹⁰⁴ Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. **Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2.** *J Biol Chem* 1999 Jun 18;274(25):17406-9
- ¹⁰⁵ Aderem A, Ulevitch RJ. **Toll-like receptors in the induction of the innate immune response.** *Nature* 2000, Aug 17;406(6797):782-787
- ¹⁰⁶ Takeuchi O, Hoshino K, Akira S. **Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection.** *J Immunol* 2000 Nov 15;165(10):5392-6.
- ¹⁰⁷ Akira S, Uematsu S, Takeuchi O. **Pathogen recognition and innate immunity.** *Cell* 2006 Feb 24;124(4):783-801.

-
- ¹⁰⁸ Tian J, Avalos AM, Mao SY, Chen B, Senthil K, Wu H, et al. **Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE.** *Nat Immunol* 2007 May;8(5):487-96.
- ¹⁰⁹ O'Neill LA, Golenbock D, Bowie AG. **The history of Toll-like receptors: redefining innate immunity** *Nat Rev Immunol* 2013 Jun;13(6):453-60
- ¹¹⁰ Takeda K, Akira S. **TLR signaling pathways.** *Semin Immunol* 2004 Feb;16(1):3–9.
- ¹¹¹ Kawai T, Akira S. **TLR signalling.** *Cell Death Differ* 2006 May;13(5):816-25.
- ¹¹² Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. **Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway.** *Science* 2003 Aug 1;301(5633):640–3 Epub 2003 Jul 10
- ¹¹³ Kawai T, Akira S. **The role of pattern-recognition receptors in innate immunity: Update on Toll-like receptors.** *Nat Immunol* 2010 May;11(5):373–84.
- ¹¹⁴ Yang Y, Kim SC, Yu T, Yi S, Rhee MH, Sung GH, Yoo BC, Cho JY. Functional roles of p38 mitogen-activated protein kinase in macrophage-mediated inflammatory responses. *Mediators Inflamm* 2014;2014:352371
- ¹¹⁵ Sinistro A, Almerighi C, Ciaprini C, Natoli S, Sussarello E, Di Fino S, Calo-Carducci F, Rocchi G, Bergamini A. **Downregulation of CD40 Ligand Response in Monocytes from Sepsis Patients.** *Clin Vaccine Immunol* 2008 Dec;15(12):1851-8
- ¹¹⁶ Gallinaro RN, Naziri W, McMasters KM, Peyton JC, Cheadle WG. **Alteration of mononuclear cell immune-associated antigen expression, interleukin-1 expression, and antigen presentation during intra-abdominal infection.** *Shock*. 1994 Feb;1(2):130-4.
- ¹¹⁷ Monneret G, Venet F, Pachot S, Lepape A. **Monitoring Immune Dysfunctions in the Septic Patient: A New Skin for the Old Ceremony.** *Mol Med* 2008 Jan-Feb; 14(1-2):64-78
- ¹¹⁸ Dong C, Davis RJ, Flavell RA. **MAP kinases in the immune response.** *Annu Rev Immunol*. 2002 ;20:55-72
- ¹¹⁹ Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH et al. **TNF- α induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway.** *Cell* 2000 Dec 22;103(7):1071-83
- ¹²⁰ Rao KMK. **MAP kinase activation in macrophages.** *J Leuk Biol*, 2001 69(1):3-10.

-
- ¹²¹ Jaworowski A, Wilson NJ, Christy E, Byrne R, Hamilton JA. **Roles of the mitogen-activated protein kinase family in macrophage responses to colony-stimulating factor-1 addition and withdrawal** *J. Biol. Chem.* 1999 274;15127-15133
- ¹²² Chan ED, Riches DW. **IFN- γ + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38 mapk in a mouse macrophage cell line.** *Am J Physiol Cell Physiol*, 2001 280(3):C441-50.
- ¹²³ Beeson PB. **Development of tolerance to typhoid bacterial pyrogen and its abolition by reticulo-endothelial blockade.** *Proc Soc Exp Biol Med.* 1946;61:248–250
- ¹²⁴ West, MA. **Endotoxin tolerance: A review** *Crit Care Med.* 2002 Jan;30(1 Supp):S64-S73
- ¹²⁵ del Fresno C, Garcia-Rio F, Gomez-Pina V, Soares-Schanoski A, Fernandez-Ruiz I, Jurado T, et al. **Potent phagocytic activity with impaired antigen presentation identifying lipopolysaccharide-tolerant human monocytes: demonstration in isolated monocytes from cystic fibrosis patients** *J. Immunol.* 2009 May 15;182(10):6494–507
- ¹²⁶ Fontaine M, Planel S, Peronnet E, Turrel-Davin F, Piriou V et al. **S100A8/A9 mRNA induction in an ex-vivo model of endotoxin tolerance: roles of IL-10 and IFN- γ .** *PLoS One* 2014 Jun 23;(6)e100909
- ¹²⁷ Foster SL, Hargreaves DC, Medzhitov R. **Gene specific control of inflammation by TLR-induced chromatin modifications.** *Nature* 2007 Jun 21;447(7147);972-8
- ¹²⁸ Fuentes-Prior P, Biswas SK, López-Collazo E, Prados C, Arnalich F, Gutierrez Del Arroyo A, García-Rio F, Gómez-Piña V, Soares-Schanoski A, Fernández-Ruiz I, Jurado T, Kajiji T, Shu C, Marín E, del Fresno C. **Potent phagocytic activity with impaired antigen presentation identifying lipopolysaccharide-tolerant human monocytes: demonstration in isolated monocytes from cystic fibrosis patients.** *J Immunol* 2009 May 15;182(10):6494-507
- ¹²⁹ Rayhane N, Fitting C, Cavaillon J-M. **Dissociation of INF γ from IL-12 and IL-8 during endotoxin tolerance.** *J Endotoxin Res.* 1999;5:319–324.
- ¹³⁰ Mathison JC, Virca GD, Wolfson E, Tobias PS, Glaser K, Ulevitch RJ. **Adaptation to bacterial lipopolysaccharide controls lipopolysaccharide-induced tumor necrosis factor production in rabbit macrophages.** *J Clin Invest.* 1990;85:1108–1118

-
- ¹³¹ Rajaiah R, Perkins DJ, Polumuri SK, Zhao A, Keegan AD, Vogel SN. **Dissociation of Endotoxin Tolerance and Differentiation of Alternatively Activated Macrophages.** *J Immunol* 2013;190:4763-4772
- ¹³² Allantaz-Frager F, Turrel-Davin F, Venet F, Monnin C, De Saint Jean A et al. **Identification of biomarkers of response to IFN γ during endotoxin tolerance: application to septic shock.** *PLoS One* 2013 Jul 11;8(7):e68218
- ¹³³ Turrel-Davin F, Venet F, Monnon C, Barbalat V, Cerrato E, Pachot A et al. **mRNA-based approach to monitor recombinant gamma-interferon restoration of LPS-induced endotoxin tolerance.** *Crit Care* 2011;15(5):r252
- ¹³⁴ Ivashkiv LB. **Inflammatory signaling in macrophages: transitions from acute to tolerant and alternative activation states** *Eur J Immunol* 2011;41:2477–81
- ¹³⁵ Adib-Conquy M, Adrie C, Moine P, Asehnoune K, Fitting C, Pinsky MR, Dhainaut J-F, Cavaillon J-M. **NF- κ B expression in mononuclear cells of septic patients resembles that observed in LPS-tolerance.** *Am J Respir Crit Care Med.* 2000;162:1877–1883



Foley, N. M. 2015. Macrophage polarisation: the impact of M1 versus M2 polarisation on host innate immune responses to bacterial infection. MD Thesis, University College Cork.

Please note that Chapters 2&3 (pp. 46-90) are unavailable due to a restriction requested by the author.

CORA Cork Open Research Archive <http://cora.ucc.ie>

Chapter 4

Discussion

Sepsis is a lethal condition. Jim Henson, the creator of The Muppets TV show died from *Streptococcus pyogenes* sepsis in 1990 at the age of 53. He developed a pneumonia, that rapidly progressed to sepsis associated multi-organ failure and consequently, death. Others who have died from sepsis include Alexander of Greece (a monkey bite), James Garfield (infected gunshot wounds), Napoleon III (gallbladder sepsis), William Hewson (a surgeon who died from sepsis after dissecting a cadaver) and Mary Wollstonecraft, an English feminist (puerperal fever). More recently, Rory Staunton, the 12-year-old son of Irish immigrants to the USA, died from undiagnosed streptococcal sepsis, which developed from an infected cut the boy sustained to his elbow during an indoor basketball match. His parents have dedicated their time to raising awareness of sepsis and instituting 'Rory's regulations' in New York City hospitals, which aim for early identification and treatment of sepsis. Despite the improved recognition of sepsis however, United States data show that cases have risen from 82.7 cases per 100,000 in 1979 to 240.4 cases per 100,000 in 2000.¹

Research into immune modulation in sepsis began in the 1960's when corticosteroids were used to dampen down the immune response in severe sepsis. However, clinical trials have failed to show a definitive benefit for the use of steroids in septic patients. Recently, a meta-analysis showed that short courses of glucocorticoids actually reduced survival in sepsis but that physiological levels of hydrocortisone

improved survival rates in patients with vasopressor-dependent shock.² A more recent study has found that evidence is still lacking to either support or refute the use of steroids at any dose in patients with sepsis.³

One of the better-known targets in the treatment of sepsis is LPS, however attempts to inject an endotoxin antiserum were unsuccessful in reducing mortality. Another molecule, TNF- α , has been targeted in clinical trials. A recent meta-analysis examining the use of anti-TNF agents in sepsis found that they produce only a modest decrease in the risk of dying from sepsis.⁴ Other molecules targeted include, IL-1, platelet activating factor and nitric oxide. Overall there have been over 100 Phase II and III clinical trials investigating compounds that target endogenous mediator molecules either in a discriminatory or non-discriminatory manner.⁵

An understudied aspect of the immune response to sepsis, are macrophages. Macrophages are amazingly diverse cells, with a unique ability to kill invading pathogens within hours. They are the first line in the host defense mechanism. M1 polarisation of macrophages is induced by priming the cells with LPS and IFN- γ , M2 activation is induced by priming cells with IL-4.⁶ Chartouni *et al* suggested that macrophages are simply primed by their respective stimuli and not activated until they come into contact with microbial stimuli.⁷ Recent research allowed for the proteomic profiling of M1 macrophages.⁸ Feng et al polarised macrophages towards an M1 and M2 polarisation profile using LPS/IFN- γ and IL-4 respectively. They found significantly

higher levels of TNF- α and IL-6 in M1 polarised macrophages compared with untreated and M2 macrophages,⁹ which is consistent with these data.

A prior study demonstrated that pre-treatment of wild type macrophages with IL-4 (the prototypic direct inducer of M2 macrophages) did not induce an endotoxin tolerant state. This is important to note, as IL-4 was used in this study as the prototypic inducer of M2 macrophages prior to treatment with bacteria. M2 macrophages are known to resemble a tolerant state, which is separate to their inducing stimuli. Dabritz et al discovered that monocytes polarised with GM-CSF, were similar to monocytes treated with IL-4 and displayed increased production of IL-1 β and TNF- α , following stimulation with LPS, compared with control monocytes.¹⁰ The polarised cells were subsequently injected into clodronate treated mice and the animals subjected to a CLP model of sepsis. Animals injected with M2 polarised macrophages had a better survival rate than animals injected with M1 polarised macrophages. This was backed up by clinical experiments in baboons in which the animals were implanted with an E. coli laden fibrin clot. Animals with a mixed M1/M2 macrophage polarisation profile, as defined by assessment of peripheral blood mononuclear cells, were found to have a survival advantage compared with those who had a prominent M1 polarisation profile.¹¹

This study sought to examine the response of polarized macrophages in response to bacterial stimulation. The concept of therapeutic macrophage manipulation in the treatment of sepsis is promising. Targeting macrophages and their polarisation profiles could allow for the immune response to be directed towards an M1 or M2 pathway. This has potential benefits in dampening down the overwhelming immune response that is

associated with sepsis and septic shock, and which is driven by M1 macrophages. It is proposed that macrophages could be isolated from a patient's own serum, and subsequently polarised to an M2 phenotype. Following this the polarised macrophages could be re-introduced into the patient. M2 macrophages have potent anti-inflammatory effects and participate in a negative feedback loop to dampen down the M1 response. An attractive potential of this type of treatment is the ability to use the body's own defenses, negating potential risks from donor sources.

The immune response to sepsis is an extraordinarily complex mechanism with multiple interconnected pathways. The concept of therapeutic macrophage manipulation should allow for all aspects of the inflammatory signaling pathways to be modified based on the polarisation profile of the exogenously polarised macrophages.

In this set of experiments, the cytokine profile of naïve and polarised macrophages was investigated at baseline and following stimulation with gram-positive and gram-negative bacteria. M1 polarised macrophages had significantly higher levels of pro-inflammatory cytokines at baseline compared with naïve and M2 polarised macrophages. Following stimulation with both gram positive and gram-negative bacteria, M1 polarised macrophages were seen to have much suppressed production of pro-inflammatory cytokines compared with naïve and M2 macrophages. These results indicated that M1 polarised macrophages exposed to bacterial stimulation were displaying an endotoxin tolerance like phenomenon.

Endotoxin tolerance is known to be a protective mechanism in which cells exposed to low concentrations of endotoxin, enter a period of hypo-responsiveness to further challenges with endotoxin. Endotoxin tolerance is associated with a reduction in the production of pro-inflammatory cytokines. However, septic patients, who develop a period of immune hypo-responsiveness after the initial cytokine storm, can display greater susceptibility to secondary infection leading to susceptibility to superimposed infections and a higher risk of death. M2 macrophages are classically associated with resolution of inflammation and tissue repair. Studies have demonstrated a possible relationship between M2 macrophage polarisation and endotoxin tolerance. Pena *et al* reported that endotoxin tolerant macrophages represented a distinct state of M2 polarisation.¹² By investigating the gene expression microarray profile among LPS treated, LPS tolerant and M2 polarised macrophages, it was found that tolerant cells have a gene expression profile more closely resembling M2 polarised macrophages.

Inflammation, or the body's response to infection, is essential in overcoming infection, and involves activation of the immune system. This inflammatory response is under strict control, with the ultimate goal involving elimination of the offending microorganism. Initially the host must sense the invading organism through pattern recognition receptors. Specific mechanisms exist for both the recognition of Gram positive and gram-negative organisms. Lipid A is a component of Gram-negative bacterial cell walls and is detected by TLR4. LTA is a component of Gram-positive bacterial cell walls and is detected by TLR2. Cytokines released following the recognition of these bacterial components, are vital effectors in directing the host innate

immune response to infection. Activated macrophages are one of the main stimuli for the release of pro-inflammatory cytokines such as TNF, IL-6 and IL-12.

An M1 polarization profile is associated with an effective immune response to bacterial infection.¹³ Flohe et al showed that IFN- γ can cause dendritic cells from septic mice to recover the ability to secrete IL-12, within 6 hours of induction. Hessle et al suggested that gram-positive bacteria have a greater capacity to induce IL-12, whereas gram negative bacteria were more likely to induce IL-10.¹⁴ Other research has shown an inhibitory effect of TNF- α on the production of IL-12p70.¹⁵ This may explain the lack of any statistical difference between IL-12p70 levels in murine macrophages exposed to *Staphylococcus aureus*. Strindhall et al, looked at various clinical isolates of staphylococcus and discovered that different isolates varied in their ability to stimulate a pro-inflammatory response in human endothelial cells.¹⁶ (These were isolates taken from individual patients e.g., patients with skin or mucosal infections, and were a mix of *S aureus* and methicillin resistant *S aureus*). In this study pure isolates of *Staph Aureus* were used, circumventing this issue.

In this study M1 macrophages had reduced production of pro-inflammatory cytokines compared with naïve and M2. This was not something that we had expected and is in contrast to previous studies. Research has shown that in septic patients, levels of IL-6, on stimulation with LPS, are severely blunted from Day 1 onwards. This suggests host immunosuppression might be responsible for the late deaths seen in patients suffering from sepsis.^{17,18} M1 macrophages are classically pro-inflammatory and microbicidal, whereas M2 macrophages are classically anti-inflammatory and play an immunomodulatory role. The typical response to bacterial infection

involves the upregulation of genes involved in M1 polarisation,¹⁹ including IL-6, TNF- α and IL-12. IL-6 is also a pro-coagulation mediator,²⁰ with coagulopathy being a sign of severe sepsis.

Previous studies have shown that M1 macrophages produce higher amounts of IL-6 on stimulation with bacteria. *Staphylococcus aureus* is able to stimulate production of IL-6 in T-cells and monocytes from whole human blood.²¹ Bost *et al.*, found high levels of IL-6 and IL-12 in murine osteoblasts infected with *Staphylococcus aureus*. *Salmonella typhi* treatment of human epithelial cells has previously been shown to be associated with elevated levels of IL-6.²² Mathur *et al* looked at the response of mice to *Salmonella typhimurium* infection and discovered elevated levels of serum IL-6 following infection which was ameliorated in TLR11 knockout mice.²³ In this study M1 macrophages had reduced production of pro-inflammatory cytokines compared with naïve and M2 macrophages. Recent studies have shown despite the M1-M2 paradigm depending on the site of inflammation a mixed population of macrophages can be present. Bystrom *et al.*, used an *in vivo* model for acute inflammation and showed that the macrophages found during the resolution phase are the same as the cells that had migrated into the inflamed site during the pro-inflammatory phase.²⁴ Further, polarisation of macrophages to M1 or M2 phenotype is more representative of a continuum state and a clear dichotomy is not always present.²⁵ In these experiments, other factors such as length of stimulation of macrophages and concentrations of stimulus may have been a factor.

The polarisation of macrophages is a highly dynamic process. Different concentrations of bacteria can elicit different cellular effects. An elegant study by Sedivy-Haley *et al.*, demonstrated that when exposed to different concentrations of bacteria, M1 polarised macrophages are relatively resistant to intracellular *Salmonella typhi*.²⁶ Moreover,

although the mechanism remains to be fully elucidated a recent study suggests that intracellular Salmonella may regulate the secretion of IL-12.

Phagocytosis assays

Phagocytosis is an important function of the initial host innate immune response to microbial infection. Macrophages undergoing M2 polarisation tend to display enhanced phagocytosis capabilities.²⁷ Tolerance to LPS may protect against bacterial infection. A study examining the fungicidal phagocytosis capabilities of endotoxin tolerant macrophages found diminished phagocytosis capabilities in tolerant macrophages.²⁸ The same study also found higher levels of NO production in the tolerant macrophages. A study from 2003 reported suppressed expression of two phagocytic receptors, CR3 and FcγIII/IIIR, in LPS tolerant murine macrophages.²⁹ This study showed equivalent phagocytosis between M1 and M2 macrophages.



Foley, N. M. 2015. Macrophage polarisation: the impact of M1 versus M2 polarisation on host innate immune responses to bacterial infection. MD Thesis, University College Cork.

Please note that Chapter 5 (pp. 99-104) is unavailable due to a restriction requested by the author.

CORA Cork Open Research Archive <http://cora.ucc.ie>

References

-
- ¹ Martin GS, Mannino DM, Eaton S and Moss M. **The epidemiology of sepsis in the United States from 1979 through 2000.** *NEJM* 2003;348:1546-1554
- ² Minneci PC, Deans KJ, Banks SM, Eichacker PQ, Natanson C. **Meta-Analysis: The effect of steroids on survival and shock during sepsis depends on the dose.** *Ann Intern Med* 2004 Jul 6;141(1):47-56
- ³ Volbeda M, Wettersley J, Gluud C, Zijlstra JG, van der Horst IC and Keus F. **Glucocorticosteroids for sepsis: sytematic review with meta-analysis and trial sequential analysis.** *Intensive Care Med* 2015 Jul;41(7):1220-34
- ⁴ Qiu P, Cui X, Sun J, Welsh J, Natanson C and Eichacker PQ. **Anti-tumour necrosis factor therapy is associated with improved survival in clinical sepsis trials: A Meta-analysis.** *Crit Care Med* 2013 Oct;41(10):2419-29
- ⁵ Marshall JC. **Why have clinical trials in sepsis failed.** *Trends Mol Med.* 2014 Apr;20(4):195-203.
- ⁶ Gordon S, Taylor PR. **Monocyte and macrophage heterogeneity.** *Nat Rev Immunol* 2005 5, 953-963
- ⁷ Chartouni CE and Rehli M. **Comprehensive analysis of TLR4-induced transcriptional responses in interleukin 4-primed mouse macrophages.** *Immunobiol* 2010 215;780-787
- ⁸ Brown J, Wallet MA, Krastins B, Sarracino D, Goodenow MM. **Proteome bioprofiles distinguish between M1 priming and activation states in human macrophages.** *J Leukoc Biol* 2010 Apr;87(4):655-62
- ⁹ Feng L, Song P, Zhou H, Li A, Ma Y, Zhang X et al. **Pentamethoxyflavanone regulates macrophage polarization and ameliorates sepsis in mice.** *Biochem Pharmacol* 2014 May 1;89(1):109-18

-
- ¹⁰ Dabritz J, Weinlage T, Varga G, Wirth T, Walscheid K et al. **Reprogramming of monocytes by GM-CSF contributes to regulatory immune functions during intestinal inflammation.** *J Immunol* 2015 Mar 1;194(5):2424-38
- ¹¹ Mehta A, Brewington R, Chatterji M, Zoubine M, Kinasewitz GT, Peer GT et al. **Infection-induced modulation of M1 and M2 phenotypes in circulating monocytes: Role in immune monitoring and early prognosis of sepsis.** *Shock* 2004 Nov;22(5):423-30
- ¹² Pena OM, Pistolic J, Raj D, Fjell CD, Hancock REW. **Endotoxin tolerance represents a distinctive state of alternative polarization (M2) in human mononuclear cells.** *J Immunol* 2011 Jun 15;186(12):7243-54
- ¹³ Flohe SB, Agrawal H, Flohe S, Rani M, Bangen JM and Schade FU. **Diversity of IFN- γ and granulocyte-macrophage colony stimulating factor in restoring immune dysfunction of dendritic cells and macrophages during polymicrobial sepsis.** *Mol Med* 2008 May-Jun;14(5-6):247-256
- ¹⁴ Hessle C, Andersson B and Wold AE. **Gram-positive bacteria are potent inducers of monocytic interleukin-12(IL-12) while gram-negative bacteria preferentially stimulate IL-10 production.** *Infect Immun* 2000 Jun;68(6):3581-3586
- ¹⁵ Ma X, Sun J, Papasavvas E, Riemann H, Robertson S, Marshall J et al. **Inhibition of IL-12 production in Human monocyte derived macrophages by TNF.** *J Immunol* 2000;164;1722-1729
- ¹⁶ Strindhall J, Lindgren PE, Lofgren S, Kihlstrom E. **Clinical isolates of Staphylococcus aureus vary in ability to stimulate cytokine expression in human endothelial cells.** *Scand J Immunol* 2005 Jan;61(1):57-62
- ¹⁷ Meakins JL, Pietsch JB, Bubenich O, Kelly R, Rode H, Gordon J et al, **Delayed hypersensitivity: indicator of acquired failure of host defences in sepsis and trauma.** *Ann Surg* 1977 Sep;186(3):241-250
- ¹⁸ Ertel W, Kremer JP, Kenney J, Steckholzer U, Jarra D et al, **Downregulation of proinflammatory cytokines in whole blood from septic patients.** *Blood* 1995 Mar 1;85(5):1341-7
- ¹⁹ Benoit M, Desnues B, Mege JL. **Macrophage polarization in bacterial infections.** *J Immunol* 2008 Sep 15;181(6):3733-9

-
- ²⁰ van der Poll T, de Jonge E, Levi M, van Deventer SJH. **Pathogenesis of DIC in Sepsis.** *Sepsis* 1999;3(2):103-9
- ²¹ Wang JE, Jorgensen PF, Almlöf M, Thiemermann C, Foster SJ, Aasen AO et al. **Peptidoglycan and Lipoteichoic acid from staphylococcus aureus induce Tumour necrosis factor alpha, Interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model.** *Infect Immun* 2000;68(7):3965-70
- ²² Weinstein DL, O'Neill BL, Metcalf ES. **Salmonella typhi stimulation of human intestinal epithelial cells induces secretion of epithelial cell derived interleukin-6.** *Infect Immun* 1997 65(2):395-404
- ²³ Mathur R, Oh H, Zhang D, Park SG, Seo J, Koblansky A et al. **A mouse model of Salmonella typhi infection.** *Cell* 2012 Oct 26;151(3):590-602
- ²⁴ Bystrom J, Evans I, Newson J, Stables M, Toor I, Van Rooijen N., et al. **Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP.** *Blood*, 2008 Nov 15;112(10):4117-27.
- ²⁵ Atri C, Guerfali FZ, Laouini D. **Role of Human Macrophage Polarization in Inflammation during Infectious Diseases.** *Int J Mol Sci.* 2018 Jun 19;19(6):1801.
- ²⁶ KJM Sedivy-Haley. **The Many Faces of Macrophages: Polarization, Endotoxin Priming, and Salmonella Resistance.** PhD. The University of British Columbia (Vancouver). April 2020 (Unpublished)
- ²⁷ Leidi ME, Gotti E, Bologna L, Miranda E, Rimoldi M, Sica A, Roncalli M, Palumbo GA, Introna M, Golay J. **M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than M1 cells in vitro.** *J Immunol.* 2009 Apr 1;182(7):4415–4422.
- ²⁸ De Lima TM, Sampaio SC, Petroni R, Brigatte P, Velasco IT, Soriano FG. **Phagocytic activity of LPS tolerant macrophages.** *Mol Immunol.* 2014 Jul;60(1):8-13
- ²⁹ Wang JH, Doyle M, Manning BJ, Blankson S, Wu QD, Power C, Cahill R, Redmond HP. **Cutting Edge: Bacterial lipoprotein induces endotoxin-independent tolerance to septic shock.** *J Immunol* 2003 Jan 1;170(1):14-18

-
- ³⁰ Lamkanfi M, Sarkar A, Vande Walle L, Vitari AC, Amer AO, Wewers MD et al., **Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia.** *J Immunol* 2010 Oct 1;185(7):4385-92.
- ³¹ Suda K, Kitagawa Y, Ozawa S, Saikawa Y, Ueda M, Ebina M, et al, **Anti-high mobility group box chromosomal protein 1 antibodies improve survival of rats with sepsis** *World J Surg* 2006 Sep;30(9):1755-62.
- ³² Opal SM, Laterre PF, Francois B, LaRosa SP, Angus DC, Mira JP, et al, ACCESS Study Group. **Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: the ACCESS randomized trial.** *JAMA* 2013 Mar 20;309(11):1154–62
- ³³ Roger T, Froidevaux C, Le Roy D, Reymond MK, Chanson AL, Mauri D, et al, **Protection from lethal gram-negative bacterial sepsis by targeting Toll-like receptor 4.** *Proc Natl Acad Sci USA* 2009 Feb 17;106(7):2348-52
- ³⁴ Daubeuf B, Mathison J, Spiller S, Hugues S, Herren S, Ferlin W, et al, **TLR4/MD2 monoclonal antibody therapy affords protection in experimental models of septic shock.** *J Immunol* 2007 Nov 1;179(9):6107-14
- ³⁵ van Lieshout MH, van der Poll T, Van't Veer C. **TLR4 inhibition impairs bacterial clearance in a therapeutic setting in murine abdominal sepsis.** *Inflamm Res* 2014 Nov;63(11):927-33
- ³⁶ Kissner TL, Moisan L, Mann E, Alam S, Ruthel G, Ulrich RG, et al. **A small molecule that mimics the BB-loop in the Toll/IL-1 receptor domain of MyD88 attenuates staphylococcal enterotoxin B-induced pro-inflammatory cytokine production and toxicity in mice.** *J Biol Chem* 2011 Sep 9;286(36):31385-96
- ³⁷ Juliana C, Fernandes-Alnemri T, Wu J, Datta P, Solorzano L, Yu JW, et al. **Anti-inflammatory compounds parthenolide and Bay 11-7082 are direct inhibitors of the inflammasome.** *J Biol Chem* 2010 Mar 26;285(13):9792-802
- ³⁸ Matsunaga N, Tsuchimori N, Matsumoto T, Ii M. **TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules.** *Mol Pharmacol* 2011 Jan;79(1):34-41.
- ³⁹ Takashima K, Matsunaga N, Yoshimatsu M, Hazeki K, Kaisho T, Uekata M, Hazeki O, Akira S, Iizawa Y, Ii M. **Analysis of binding site for the novel small- molecule TLR4**

signal transduction inhibitor TAK-242 and its therapeutic effect on mouse sepsis model. *Br J Pharmacol* 2009 Aug;157(7):1250-62.

⁴⁰ Sha T, Sunamoto M, Kitazaki T, Sato J, Ii M, Iizawa Y. **Therapeutic effects of TAK-242, a novel selective Toll-like receptor 4 signal transduction inhibitor, in mouse endotoxin shock model.** *Eur J Pharmacol* 2007 Oct 1;571(2-3):231-39.

⁴¹ Sha T, Iizawa Y, Ii M, **Combination of Imipenem and Tak-242, a Toll-like receptor 4 signal transduction inhibitor, improves survival in a murine model of polymicrobial sepsis.** *Shock* 2011 Feb;35(2):205-9

⁴² Takeda [http://www.takeda.com/news/2009/20090220_3678.html].

⁴³ Rice T, Wheeler A, Bernard G, Vincent JL, Angus D, Aikawa N, Demeyer I, Sainati S, Amlot N, Cao C, Ii M, Matsuda H, Mouri K, Cohen J. **A randomized, double-blind, placebo-controlled trial of TAK-242 for the treatment of severe sepsis.** *Crit Care Med* 2010 Aug;38(8):1685-94

⁴⁴ Meng G, Rutz M, Schiemann M, Metzger J, Grabiec A, Schwandner R, Lippa PB, Ebel F, Busch DH, Bauer S, Wagner H, Kirschning CJ. **Antagonistic antibody prevents Toll-like receptor 2-driven lethal shock-like syndromes.** *J Clin Invest* 2004 May;113(10):1473–81.

⁴⁵ Spiller S, Elson G, Ferstl R, Dreher S, Mueller T, Freudenberg M, Daubeuf B, Wagner H, Kirschning CJ. **TLR4-induced IFN-gamma production increases TLR2 sensitivity and drives gram-negative sepsis in mice.** *J Exp Med* 2008 Aug 4;205(8):1747-54

⁴⁶ Cavassani KA, Ishii M, Wen H, Schaller MA, Lincoln PM, Lukacs NW, Hogaboam CM, Kunkel SL. **TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events.** *J Exp Med* 2008 Oct 27;205(11):2609–21

⁴⁷ Wang Y, Wang YP, Zheng G, Lee VWS, Ouyang L, Chang DHH et al. **Ex Vivo programmed macrophages ameliorate experimental chronic inflammatory renal disease.** *Kidney Int* 2007 Aug;72(3):290-9

⁴⁸ Hunter MM, Wang A, Parhar KS, Johnston MJG, van Rooijen N, Beck PL et al. **In vitro-derived alternatively activated macrophages reduce colonic inflammation in mice.** *Gastroenterol* 2010 Apr;138(4):1395-405

⁴⁹ Fink MP. **Animal models of sepsis.** *Virulence* 2014, Jan1;5(1):143-53

-
- ⁵⁰ Van Rooijen N, Sanders A. **Elimination, blocking and activation of macrophages: three of a kind?** *J Leuk Biol* 1997 Dec;62(6):702-9.
- ⁵¹ Traeger T, Mikulcak M, Eipel C, Abshagen K, Diedrich S, Heidecke CD et al. **Kupffer cell depletion reduces hepatic inflammation and apoptosis but decreases survival in abdominal sepsis.** *Eur J Gastroenterol Hepatol* 2010 Sep;22(9):1039-49
- ⁵² Traeger T, Kessler W, Hilpert A, Mikulcak M, Entleutner M, Koerner P et al. **Selective depletion of alveolar macrophages in polymicrobial sepsis increases lung injury, bacterial load and mortality but does not affect cytokine release.** *Respiration* 2009;77(2):203-13
- ⁵³ Claassen I, Van Rooijen N, Claassen E. **A new method for removal of mononuclear phagocytes from heterogeneous cell populations in vitro, using the liposome-mediated macrophage ‘suicide’ technique.** *J Immunol Methods*. 1990 Dec 5;134(2):153-61
- ⁵⁴ Naito M, Nagai H, Kawano S, Umezu H, Zhu H, Moriyama H et al. **Liposome-encapsulated dichloromethylene diphosphonate induces macrophage apoptosis in vivo and in vitro.** *J Leukoc Biol*.1996 Sep;60(3):337-44.
- ⁵⁵ Koay MA, Gao X, Washington MK, Parman KS, Sadikot RT, Blackwell TS et al. **Macrophages are necessary for maximal nuclear factor- κ B activation in response to endotoxin challenge.** *Am J Respir Cell Mol Biol* 2002 May;26(5):572-8
- ⁵⁶ Sica A, Sacconi A, Bottazzi B, Polentarutti N, Vecchi A, Van Damme J et al. **Autocrine production of IL-10 mediates defective IL-12 production and NF- κ B activation in tumour-associated macrophages.** *J Immunol* 2000 Jan 15;164(2):762-7
- ⁵⁷ Mosser DM, Edwards JP. **Exploring the full spectrum of macrophage activation.** *Nat Rev Immunol*. 2008 Dec;8(12):958-969
- ⁵⁸ Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J. **Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences.** *J Immunol* 2005 Jul 1;175(1):342-9
- ⁵⁹ Parsa R, Andresen P, Gillett A, Mia S, Zhang XM, Mayans S et al. **Adoptive transfer of Immunomodulatory M2 macrophages prevents type 1 diabetes in NOD mice.** *Diabetes* 2012 Nov;61(11):2881-92

⁶⁰ Smith TD, Tse MJ, Read EL, Liu WF. **Regulation of macrophage polarization and plasticity by complex activation signals.** *Integr Biol (Camb)* 2016 Sep 12;8(9):946–55.